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Rapid detection of inducers of enzymes that protect against carcinogens

(cancer/chemoprotection/enzyme induction/quinone reductase/vegetables)

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ABSTRACT Dietary composition is a major determinant of cancer risk in humans and experimental animals. Major and minor components of the diet may enhance or suppress the development of malignancy. Many dietary constituents also modify the metabolism of carcinogens by induction of enzymes involved in xenobiotic metabolism, and this is one well-established mechanism for modulating the risk of cancer. We have developed a simple system for rapid detection and measurement of the induction of enzymes that detoxify carcinogens (phase II enzymes), based on the direct assay of the activity of quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2] in murine hepatoma cells grown in microtiter plate wells. Survey of extracts of a variety of commonly consumed, organically grown vegetables for quinone reductase inducer activity identified crucifers (and particularly those of the genus *Brassica*) as singularly rich sources. It is therefore of interest that high consumption of these types of vegetables has been correlated with decreased cancer risk in humans. The assay system also measures toxicity, which was unrelated to inducer potency among the vegetable extracts examined. By use of mutant hepatoma cells (defective in regulation of certain cytochrome P-450 enzymes) selective (monofunctional) inducers of protective phase II enzymes can be distinguished from (bifunctional) inducers that also elevate cytochromes P-450 (phase I enzymes) and thereby pose the risk of carcinogen activation. The assay system therefore permits not only rapid detection of inducers of anticarcinogenic enzymes in the human diet but also elucidation of effects of storage and processing on inducer activities.

Extrinsic factors, including personal life-styles, play a major role in the development of most human malignancies (1-3). Cigarette smoking and consumption of alcohol, exposure to synthetic and naturally occurring carcinogens, radiation, drugs, infectious agents, and reproductive and behavioral practices are now widely recognized as important contributors to the etiology of cancer. But perhaps most surprising is the inference that normal human diets play causative roles in more than one-third (and possibly even two-thirds) of human neoplasia (1-3). Our food contains not only numerous mutagens and carcinogens but also a variety of chemicals that block carcinogenesis in animal models (4-11). Furthermore, carcinogens can even protect against their own toxic and neoplastic effects or those of other carcinogens—i.e., carcinogens may act as anticarcinogens (12-14). Clearly, dietary modifications modulate cancer risk in various ways: for instance, through changes in caloric intake, by altering the consumption of nutritive and nonnutritive major components, and by providing exposure to numerous minor chemicals that may be genotoxic or protective (4-7, 9-11, 15-19). Rational recommendations for modifying human diets to

reduce the risk of cancer require identification of dietary carcinogens and chemoprotectors, even though interactions among such factors in the etiology of cancer are complex (20). Whereas extensive efforts have been made to identify dietary carcinogens and mutagens (4-6), chemoprotective components have received far less attention. This paper describes a method for detecting and identifying anticarcinogenic components in human diets.

Since a major mechanism regulating neoplasia is the balance between phase I enzymes, which activate carcinogens, and phase II enzymes[‡] (25, 26), which detoxify them, we have developed a cell culture system for simple and rapid detection of dietary components that enhance phase II detoxication enzymes. With this procedure we surveyed extracts of a variety of vegetables for their ability to induce such protective enzymes. In the accompanying paper (27) we describe use of this method to isolate and identify a major inducer of protective enzymes from broccoli.

We chose vegetables as sources of inducers of detoxication enzymes for the following reasons. First, numerous epidemiological studies suggest that high consumption of yellow and green vegetables, especially those of the family Cruciferae (mustards) and the genus *Brassica* (cauliflower, cress, brussels sprouts, cabbage, broccoli), reduces the risk of developing cancer of various organs (28-34). Moreover, administration of vegetables or of some of their chemical components to rodents also protects against chemical carcinogenesis (9-11, 35). Second, well-documented evidence established that feeding of certain vegetables (e.g., brussels sprouts and cabbage) induces both phase I and phase II enzymes in animal tissues (36-44) and stimulates the metabolism of drugs in humans (36, 45, 46). The elevations of enzymes that metabolize xenobiotics may be highly relevant to the protective effects of vegetables, since relatively modest dietary changes not only affected the metabolism of drugs (44) but also modified the ability of carcinogens to cause tumors in rodents (15-19, 47-49).

Several lines of evidence provide compelling support for the proposition that induction of enzymes of xenobiotic

Abbreviation: QR, quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2].

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[‡]Enzymes of xenobiotic metabolism belong to two families: (i) phase I enzymes (cytochromes P-450), which functionalize compounds, usually by oxidation or reduction; although their primary role is to detoxify xenobiotics, several cytochromes P-450 can activate procarcinogens to highly reactive ultimate carcinogens (21); and (ii) phase II enzymes, which conjugate functionalized products with endogenous ligands (e.g., glutathione, glucuronic acid, sulfate) and serve primarily a detoxication role (22). Quinone reductase (QR) is considered a phase II enzyme because it has protective functions (23), is induced coordinately with other phase II enzymes, and is regulated by enhancer elements similar to those that control glutathione transferase (24).

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metabolism, and particularly phase II enzymes, results in protection against the toxic and neoplastic effects of carcinogens (25, 26): (i) Many seemingly unrelated compounds (including phenolic antioxidants, coumarins, cinnamates, 1,2-dithiole-3-thiones, isothiocyanates, lactones, thiocarbamates) can protect rodents against the effects of carcinogens under conditions that invariably evoke the induction of phase II enzymes in many tissues. Indeed, novel anticarcinogens have been isolated and identified solely on the basis of their ability to induce phase II enzymes (50, 51). (ii) Such anticarcinogens alter the metabolism of carcinogens and decrease the formation of mutagenic metabolites (52–54). (iii) Chemoprotection requires protein synthesis and is most effective if it precedes carcinogen challenge (12–14). (iv) Inducers of anticarcinogenic enzymes protect against a wide variety of structurally dissimilar carcinogens, suggesting the involvement of mechanisms that are not structurally fastidious, such as xenobiotic metabolism. (v) The enzymes that are elevated, e.g., glutathione transferases, quinone reduc-

tase [QR; NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2], UDP-glucuronosyltransferases, protect against the toxicities of electrophiles such as ultimate carcinogens. (vi) Cells in which glutathione transferases are elevated (by development of resistance to alkylating chemotherapeutic agents or by transfection with cloned enzymes) show decreased susceptibility to the toxicity of carcinogenic electrophiles and reduced formation of DNA adducts (55–57).

Resolution of the issue whether the anticarcinogenic effects of vegetables are mediated through the induction of enzymes of xenobiotic metabolism requires the systematic bioassay of these plants for inducer activity. Since measurement of enzyme induction in animals is laborious and expensive, we developed a simple screening procedure in which the specific activity of QR, a phase II enzyme,[†] is measured in Hepa 1c1c7 murine hepatoma cells (58, 59). The feasibility of measuring inducer activity directly in cells grown in 96-well microtiter plates has simplified and accelerated the procedure (60), and the use of heat- and charcoal-treated serum in-

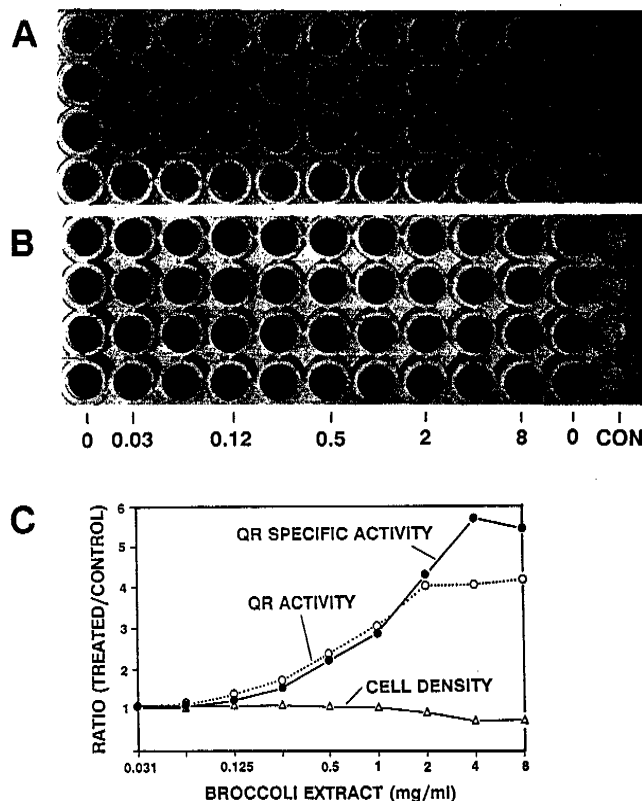


FIG. 1. Induction of QR in murine hepatoma cells by extracts of broccoli. (A and B) Photographs of sections of 96-well microtiter plates showing the induction of QR (A) and the cell density (B). (C) Graph showing the analysis of absorbances obtained from the plates. The assays were carried out on Hepa 1c1c7 murine hepatoma cells grown in microtiter plate wells and induced with serial 2-fold dilutions of acetonitrile extracts of lyophilized broccoli (Effie May). Details of the procedures are given in *Materials and Methods*. (A) The QR activities were measured in cell lysates by reduction of a tetrazolium dye. Note that the color (blue-brown) increases in intensity with the concentration of extract, indicating QR induction. (B) A parallel plate treated with the same dilutions of broccoli extract. The cells were stained with crystal violet. Note that there is a slight decrease in cell density at the highest concentrations (4–8 mg/ml), indicating mild cytotoxicity. (C) Graphical analysis of optical density information obtained from the above plates scanned at 610 nm (QR assay) and 490 nm (crystal violet assay) related to control wells that received the equivalent volume of acetonitrile only (0.2%). The total and specific activities of QR and the cell densities, expressed as ratios (treated/control), are shown on the ordinate. The concentrations of broccoli extract, shown below the designated microtiter plate wells and on the abscissa of the graph, are expressed as the amount of extract obtained from a given dry weight of broccoli (mg) added to each ml of culture medium (0–8 mg/ml). The QR activity and crystal violet density are related to cells that did not receive inducer. The columns of wells designated 0 mg/ml received no broccoli extract. CON designates the wells that contained no cells and served as the optical controls.

creased its sensitivity (61). These cells respond to nearly all compounds that induce phase II enzymes (e.g., QR and glutathione transferases) in rodents, and conversely induction of QR in these cells is a reliable predictor of inducer activity in various rodent organs *in vivo* (27, 61–63).

MATERIALS AND METHODS

Sources of Vegetables and Preparation of Extracts. All vegetables were grown under organic conditions without pesticides or artificial fertilizers that might contain enzyme inducers. They were stored at -20°C after arrival in our laboratory, although the intervening storage history of some vegetables is not known. Vegetables were homogenized with 2 vol of cold water in a Waring Blender for 2 min at 4°C . The resultant soups were lyophilized to give dry powders, which were stored at -20°C . Portions (400 mg) of these powders were extracted for 6–24 hr with 14 ml of acetonitrile by shaking in glass vessels at 4°C . The extracts were filtered through $0.45\ \mu\text{m}$ porosity organic solvent-resistant filters and evaporated to dryness either in a vacuum centrifuge (Speed-Vac; Savant) or on a rotating evaporator ($<40^{\circ}\text{C}$). The residues were dissolved or suspended in $100\ \mu\text{l}$ of acetonitrile.

Assay of Inducer Potency. Inducer activity was measured in Hepa 1c1c7 murine hepatoma cells grown in 96-well microtiter plates (60, 61). Duplicate plates were prepared. In a typical experiment 10,000 Hepa 1c1c7 cells were introduced into each well initially, grown for 24 hr, and then induced for 48 hr by exposure to medium containing serial dilutions of the extracts (or compounds) to be assayed. Usually $20\ \mu\text{l}$ of the acetonitrile solutions to be assayed were added to $10.0\ \text{ml}$ of medium and 2-fold serial dilutions were made in the microtiter plates so that the final volume in each well was $150\ \mu\text{l}$ and the organic solvent concentration was 0.2%. QR activity (based on the formation of the blue-brown reduced tetrazolium dye) was measured with an optical microtiter plate scanner in cell lysates prepared in one plate, and the cell density was determined in the second plate by staining with crystal violet. Quantitative information on specific activity of QR, the inducer potency, and the cytotoxicity of the extract or compound tested is obtained by computer analysis of the absorbances (see Fig. 1). One unit of inducer activity is defined as the amount that when added to a single microtiter well doubled the QR specific activity.

RESULTS AND DISCUSSION

Fig. 1 illustrates the measurement of inducer potency of extracts of organically grown broccoli (Effie May variety). The specific activities of QR were raised nearly 6-fold at the highest extract concentrations tested, at which less than 20% cytotoxicity was observed. The inductions obtained with broccoli (Fig. 1) and with other vegetable extracts (Fig. 2) were proportional to the quantity of extract added over a reasonably wide range. The toxicities of these extracts were modest and were unrelated to their inducer potencies (Fig. 2).

Extracts of a series of organically grown vegetables cultivated under a variety of conditions showed large differences in inducer potencies (Table 1). Because the dry weight content of the vegetables varied considerably, from 3.6% for a sample of bok choy to 26.5% for red leaf lettuce (mean 10% for 24 vegetables) (Table 1), we express the inducer activity of an extract in terms of the dry weight of vegetable yielding, upon extraction under standardized conditions, a given amount of inducer activity. This provides a measure of inducer potency, expressed as units per g of dry vegetable weight (see *Materials and Methods* for definition of unit). Although many vegetable extracts induced QR, certain families were consistently more potent inducers. For example,

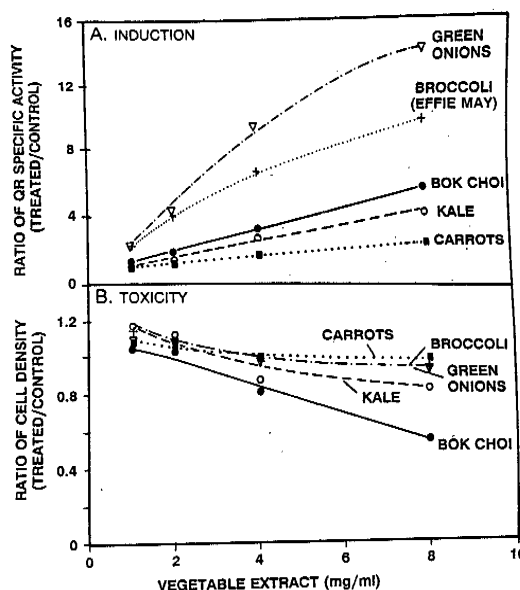


Fig. 2. Potency of induction of QR and toxicity of acetonitrile extracts obtained from five organically grown lyophilized vegetables (green onion, broccoli, bok choy, kale, and carrots) measured by the microtiter plate assay in Hepa 1c1c7 murine hepatoma cells. The extracts were prepared and assayed as described in Fig. 1 legend and *Materials and Methods*. The concentrations of extracts are expressed as the amount of extract per ml of culture medium derived from the indicated weight of dried vegetable. (A) Ratio of the specific activities of QR of treated to control cells. (B) Relative cell densities as determined by crystal violet staining measured at 490 nm. Note that the inductions are reasonably proportional to the amount of extract at lower induction ratios and that the inducer potencies and toxicities (which do not exceed 20% except in the case of bok choy) are not correlated.

whereas extracts of several Cruciferae had potent inducer activity, extracts of Solanaceae (peppers, potatoes, tomatoes) had low inducer activity. Of the 24 vegetables examined only 6 showed detectable toxicity; the others were nontoxic at the highest concentrations tested. Thus cytotoxicity of 20% was observed for red leaf lettuce at $8.0\ \text{mg/ml}$, for beets, cauliflower, and bok choy at $4.0\ \text{mg/ml}$, and for leeks and ginger at $2.0\ \text{mg/ml}$.

Cytotoxicity measurements are important because phase II enzyme inducers may be toxic and/or carcinogenic. Moreover, by use of mutant Hepa cells defective in aryl hydrocarbon receptor or cytochrome P-450 function (27, 63, 64), our assay system can distinguish *monofunctional* inducers (which elevate phase II enzymes selectively), from *bifunctional* inducers (which elevate both phase I and phase II enzymes) (63). Such information is crucial for identification of chemoprotective enzyme inducers for potential use in humans. Ideally such inducers should be monofunctional, because elevated activities of phase I enzymes may lead to carcinogen activation.

Since some crucifers (broccoli, brussels sprouts, cauliflower, cabbage) are consumed in substantial quantities in Western diets and are believed to protect against cancer, we examined the relation of inducer potency to variety, strain, location of growth, time of sowing, and time of harvest (Table 2). Although systematic examination of these factors under field conditions would require extensive studies over several

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Table 1. Potency of induction of QR in Hepa 1c1c7 murine hepatoma cells by acetonitrile extracts of representative samples of various vegetable families and species

Vegetable		Dry weight, %	Potency of QR induction, units*/g
Family	Species/variety		
Chenopodiaceae	Beets	17.3	<833
	Spinach	6.8	1,280
Compositae	Red leaf lettuce	26.5	3,030
Cruciferae	Cauliflower	9.8	2,220
	Bok choy	3.6	3,170
	Broccoli (Effie May)	10.2	16,700
	Broccoli (Winchester)	8.0	2,380
	Green cabbage	9.4	1,550
	Kale	9.2	2,220
	Radish	3.7	1,040
Cucurbitaceae	Zucchini	5.5	<833
Leguminosae	Green beans	6.7	2,150
	Sugar snap peas	14.5	<833
Liliaceae	Asparagus	5.6	1,110
	Green onions	5.1	22,200
	Leeks	8.3	2,780
Rosaceae	Apple	13.1	Inactive
Solanaceae	Green peppers	6.4	Inactive
	Red potatoes	15.7	Inactive
	Sweet potatoes	20.2	Inactive
	Tomatoes	6.2	<833
Umbelliferae	Carrots	10.8	1,230
	Celery	4.5	1,630
Zingiberaceae	Ginger	13.1	4,440

*One unit of inducer activity is defined as the amount of inducer required to double the QR specific activity of Hepa 1c1c7 cells growing in a microtiter well containing 150 μ l of medium. An entry of <833 units/g indicates that at the highest concentration tested (extract from 1.2 mg of dry vegetable/150 μ l medium) the QR specific activity was significantly elevated but not doubled. Inactive indicates less than 20% elevation of QR specific activity at highest concentration tested: extract from 1.2 mg of dry weight per 150 μ l of medium.

years of cultivation, it was important to determine whether such variables significantly affected the inducer activity. Except for a sample of kohlrabi, Cruciferae belonging to the species *Brassica oleracea* consistently and potentially induced QR (Table 2), with broccoli and brussels sprouts generally the most potent inducers. The inductive capacity of most crucifers appears to be independent of geographic location of growth and time of harvest, although late sowing may have enhanced modestly the potency of the induction. On the basis of these results a particular variety of broccoli (SAGA) was selected for isolation and identification of monofunctional inducer activity as described in the accompanying paper (27).

In summary, epidemiological studies point to the inverse relationship between vegetable consumption and the risk of epithelial cancer, and they suggest a practical approach to achieving protection by emphasizing that the typical Western diet is low in fruits and vegetables (20). A striking but perhaps not surprising conclusion is that the microtiter plate assay for induction of QR identifies the same vegetables (crucifers) that display protective properties *in vivo* (9–11, 28–35). It is critical to our understanding of the relationship of diet to cancer, however, that we assess dietary constituents not only for their abilities to induce anticarcinogenic enzymes but also for their toxic and carcinogenic properties. The simple and rapid assay can also determine the toxicity of extracts and, by use of appropriate mutant cells, distinguish monofunctional inducers from less desirable bifunctional ones. Moreover, the assay of phase II enzymes makes possible further detailed analysis of the effects of treatment of vegetables (e.g.,

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Table 2. Potency of induction of QR in Hepa 1c1c7 murine hepatoma cells by acetonitrile extracts of various cruciferous vegetables of the species *Brassica oleracea*

Vegetable (<i>B. oleracea</i> subspecies and variety)	Dry weight, %	Potency of QR induction, units/g
Broccoli (<i>B. oleracea italica</i>)		
Florets		
Emperor	9.6	6,670
Emperor (late sowing)	9.9	22,200
Green Valiant	10.4	16,700
Green Valiant (late sowing)	8.8	16,700
SAGA (older sample)	6.8	4,170
SAGA (younger sample)	8.0	11,100
SAGA (late sowing)	10.1	33,300
Violet Queen	9.6	3,700
Leaves only		
Emperor	7.0	5,560
SAGA (older sample)	9.4	3,030
SAGA (younger sample)	10.0	16,700
Violet Queen	10.6	7,410
Brussels sprouts (<i>B. oleracea gemnifera</i>)		
Jade Cross E	10.8	11,100
Oliver	15.6	6,060
Teal	14.0	11,100
Green cabbage (<i>B. oleracea capitata</i>)		
Nagoda 50	4.6	2,560
Perfect Ball	7.6	5,560
Primax	5.6	2,080
Red cabbage (<i>B. oleracea capitata</i>)		
Lasso Red	11.2	13,300
Ruby Perfection	7.0	4,760
Cauliflower (<i>B. oleracea botrytis</i>)		
Florets		
Andes	8.8	5,560
Montano	8.0	3,700
Leaves only		
Montano	7.4	3,330
Snow Crown	6.6	2,780
Kale (<i>B. oleracea acephala</i>)		
Konserva	10.4	3,170
Winterbor	8.4	4,760
Winterbor (late sowing)	15.4	16,700
Kohlrabi (<i>B. oleracea gongyloides</i>)		
Capri	5.2	1,330
Kolpak (late sowing)	6.0	1,590

The assays were performed as described in the text and legend to Table 1.

growth, storage, and cooking conditions) that might enhance or depress such induction, and also more definitive examination of the relationship of induction to chemoprotection.

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Biochemistry

Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates

(chemoprotection/quinone reductase/enzyme induction/dimethylbenzanthracene/rat mammary tumors)

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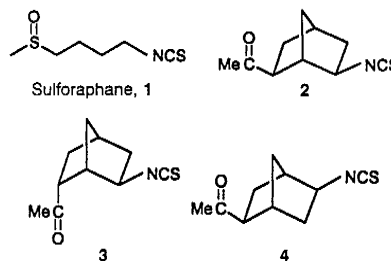
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ABSTRACT Sulforaphane [1-isothiocyanato-4-(methylsulfinyl)butane] was recently isolated from one variety of broccoli as the major and very potent inducer of phase 2 detoxication enzymes in murine hepatoma cells in culture. Since phase 2 enzyme induction is often associated with reduced susceptibility of animals and their cells to the toxic and neoplastic effects of carcinogens and other electrophiles, it was important to establish whether sulforaphane could block chemical carcinogenesis. In this paper we report that sulforaphane and three synthetic analogues, designed as potent phase 2 enzyme inducers, block the formation of mammary tumors in Sprague-Dawley rats treated with single doses of 9,10-dimethyl-1,2-benzanthracene. The analogues are *exo*-2-acetyl-*exo*-6-isothiocyanatonorbornane, *endo*-2-acetyl-*exo*-6-isothiocyanatonorbornane, and *exo*-2-acetyl-*exo*-5-isothiocyanatonorbornane. When sulforaphane and *exo*-2-acetyl-*exo*-6-isothiocyanatonorbornane were administered by gavage (75 or 150 μ mol per day for 5 days) around the time of exposure to the carcinogen, the incidence, multiplicity, and weight of mammary tumors were significantly reduced, and their development was delayed. The analogues *endo*-2-acetyl-*exo*-6-isothiocyanatonorbornane and *exo*-2-acetyl-*exo*-5-isothiocyanatonorbornane were less potent protectors. Thus, a class of functionalized isothiocyanates with anticarcinogenic properties has been identified. These results validate the thesis that inducers of phase 2 enzymes in cultured cells are likely to protect against carcinogenesis.

Enzymes that metabolize xenobiotics play a major role in regulating the toxic, mutagenic, and neoplastic effects of chemical carcinogens. Much evidence indicates that the activities of phase 2 detoxication enzymes (e.g., glutathione transferases, NAD(P)H:quinone reductase, UDP-glucuronosyltransferases, and epoxide hydrolase) in particular can modulate the response of animals and their cells to carcinogen exposure. Induction of these enzymes by a wide variety of chemicals (including components of the diet) results in protection against toxicity and neoplasia (1). To identify such protective inducers and to measure their potencies, a simple cell culture system has been developed in our laboratory (2, 3). This system depends on determining the specific activities of quinone reductase in murine hepatoma cells grown in 96-well microtiter plates and exposed to a range of concentrations of the inducers. Such measurements not only have reliably predicted the ability of compounds to induce phase 2 enzymes in rodent tissues *in vivo* but also have identified several chemoprotectors against carcinogenesis. By use of this system, sulforaphane [(*-*)-1-isothiocyanato-4-(methylsulfinyl)butane, 1] was recently isolated from Saga broccoli as the major phase 2 enzyme inducer present in organic solvent extracts of this vegetable.

Sulforaphane is of interest for three reasons: (i) it occurs naturally in a widely consumed vegetable; (ii) it is a very potent inducer of phase 2 enzymes; and (iii) it is a monofunctional inducer (4)—i.e., it elevates phase 2 detoxication enzymes without significantly changing the synthesis of cytochromes P-450 (5). These findings allowed the design and the systematic synthesis of a large number of structurally related isothiocyanates (6). It was found that the methylsulfinyl (CH_3SO) function of sulforaphane could be replaced by a methylcarbonyl (i.e., acetyl) group without significantly affecting inducer potency and that, in the most potent inducers, the isothiocyanate function and the acetyl group were separated by three or four carbons of an aliphatic or cyclo-aliphatic chain. Several isomeric norbornyl isothiocyanates substituted with acetyl groups were found to approach or equal the potency of sulforaphane as a phase 2 enzyme inducer (6). The merits of these norbornyl isothiocyanates [*exo*-2-acetyl-*exo*-6-isothiocyanatonorbornane, 2; *endo*-2-acetyl-*exo*-6-isothiocyanatonorbornane, 3; and *exo*-2-acetyl-*exo*-5-isothiocyanatonorbornane, 4] are that they can be more easily synthesized (from commercial 2-acetyl-5-norbornene) than sulforaphane and that they are probably more stable toward chemical and biological oxidation-reduction reactions.

We report here that sulforaphane and synthetic cyclic isothiocyanate analogues block mammary tumor development in Sprague-Dawley rats treated with 9,10-dimethyl-1,2-benzanthracene (DMBA) (7, 8). These findings identify a class of functionalized isothiocyanates as enzyme inducers that block carcinogenesis, and further strengthen the view that the search for phase 2 enzyme inducer activity from natural sources can successfully identify chemoprotectors against cancer (1-3). These results also encourage further efforts at rational design and laboratory synthesis of even more potent chemoprotectors.



MATERIALS AND METHODS

Animals and Mammary Tumor Development. Female Sprague-Dawley rats were obtained from Harlan-Sprague-

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Abbreviation: DMBA, 9,10-dimethyl-1,2-benzanthracene.
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Dawley Laboratories at 40 days of age. They were housed in plastic cages (four to five per cage) on Betachip hardwood laboratory bedding (Northeastern Product, Warrensburg, NY) and were fed unrestricted amounts of water and a pelleted AIN-76A diet containing no ethoxyquin (Teklad, Madison, WI). The temperature was 25°C, and 12-hr light/12-hr dark cycles were maintained. All animal experiments were in compliance with National Institutes of Health Guidelines (9) and were approved by the Animal Care and Use Committee of The Johns Hopkins University School of Hygiene and Public Health. The rats were assigned randomly to seven groups: a control group of 25 animals and six treatment groups of 20 animals each. The animals were weighed individually at weekly intervals. At age 47, 48, 49, 50, and 51 days; each animal received by gavage either 0.5 ml of Emulphor EL-620 (Rhone-Poulenc, Cranbury, NJ) alone or the specified doses (75, 100, or 150 μ mol daily) of sulforaphane (1) or compound 2, 3, or 4 in 0.5 ml of Emulphor EL-620. On day 50, 3 hr after administration of the vehicle or protector, all rats also received an intragastric instillation of 8.0 mg of DMBA (Sigma) dissolved in 1.0 ml of sesame oil. This dose of DMBA was selected to produce a substantial tumor incidence, but not one so high as to overwhelm a potential chemoprotective effect. The animals were examined once weekly for the appearance and location of palpable tumors. At age 202 days, i.e., 152 days after carcinogen administration, all animals were euthanized with ether and weighed. The tumors were separated from fat and connective tissue by dissection, weighed, and fixed in buffered 10% formalin. All tumors were identified microscopically by examination of stained sections.

Quantitative Assessment of Tumor Development. Of the 145 rats initially assigned to this experiment, 8 did not survive until we terminated the experiment when the rats were 202 days old. The deaths were distributed among the groups as follows: controls, 2 deaths with tumors; sulforaphane (150- μ mol dose), 1 death from gavage accident; sulforaphane (75- μ mol dose), no deaths; compound 2 (150- μ mol dose), 1 death with tumors; compound 2 (75- μ mol dose), 1 death with tumors; compound 3 (100 μ mol), 3 deaths, 2 with tumors, 1 without tumors; compound 4 (100 μ mol), no deaths.

Since tumor and body weights were not measured on the animals that died during the course of the experiment, we report separately the tumor incidence and multiplicity for all rats (two rats were excluded for reasons given in Table 1) and

for the 137 rats surviving to the termination of the experiment (Tables 1 and 2, respectively).

The mean body weights (\pm SEM) of the animal groups at the beginning of treatment (age 47 days) were between 116 ± 1.7 and 126 ± 1.9 g. The final weights at termination of the experiment are given in Table 2.

The development and characteristics of tumors in each group were assessed in four ways: (i) *incidence*, the fraction (percent) of animals that developed tumors; (ii) *multiplicity*, the total number of tumors divided by the total number of animals at risk; (iii) *total number and weight of tumors* removed from each animal at the termination of the experiment; and (iv) *latency of tumor development*. The proportions of tumor-free animals in the control and each treatment group were compared at the time of the weekly examinations of the animals (Fig. 1).

Statistical Analysis of Results. Differences in tumor incidence were evaluated by the Fisher exact test. Tumor multiplicity differences were analyzed by a Poisson distribution model and average rates were compared. The overall progression of tumor development was assessed by Kaplan-Meier analyses followed by logarithmic rank tests.

Chemical Syntheses. The synthetic methods and characterization of the compounds have been described (6). Multigram quantities of compounds 2–4 were prepared in one step from commercial 2-acetyl-5-norbornene (a mixture of *exo* and *endo* isomers) obtained from Aldrich.

RESULTS

Administration of sulforaphane or of the 2-acetylnorbornyl isothiocyanates 2, 3, and 4 reduced the incidence, multiplicity, and weights and delayed the development of the mammary tumors evoked by a single dose of DMBA in female Sprague-Dawley rats (Tables 1 and 2; Fig. 1). There were clear-cut differences in the potencies of the chemoprotective compounds.

In the control group, not receiving any protector, the incidence of mammary tumors for all animals was 68.0% (Table 1). If the two animals that did not survive to the termination of the experiment (rat age, 202 days) were censored, the tumor incidence in the control group was very similar, 65.2% (Table 2). The corresponding tumor multiplicities (total number of tumors per number of animals at risk) were 1.56 (all animals) and 1.43 (survivors to termination),

Table 1. Protective effects of sulforaphane and norbornyl isothiocyanates 2, 3, and 4 on incidence and multiplicity of mammary tumors in DMBA-treated female Sprague-Dawley rats

Treatment group	No. of rats		Tumor incidence, % (% of control)	No. of tumors	
	In group	With tumors		Total	Multiplicity (% of control)
Control	25	17	68.0 (100)	39	1.56 (100)
Sulforaphane					
75 μ mol	20	7	35.0* (51.4)	9	0.45† (28.8)
150 μ mol	19‡	5	26.3* (38.7)	5	0.26† (16.7)
Compound 2					
75 μ mol	20	5	25.0* (36.8)	6	0.30† (19.2)
150 μ mol	20	5	25.0* (36.8)	7	0.35† (22.4)
Compound 3 (100 μ mol)	19§	9	47.3 (69.6)	14	0.74† (47.4)
Compound 4 (100 μ mol)	20	8	40.0 (58.8)	8	0.40† (25.6)

A total of 145 rats were entered into the experiment. Each received 8 mg of DMBA at age 50 days. There were initially 25 controls and 20 animals in each of the six treated groups. The above analysis is based on 143 animals (see below).

* $P < 0.05$ for differences from controls (Fisher exact test).

† $P \leq 0.01$ for differences from controls (Poisson distribution model).

‡One rat died immediately after gavage and is not included.

§One rat died without palpable tumors at age 167 days and is not included.

Table 2. Protective effects of sulforaphane and norbornyl isothiocyanates 2, 3, and 4 on incidence, multiplicity, and weights of mammary tumors in DMBA-treated female Sprague-Dawley rats: Analysis of survivors to termination of the experiment at rat age 202 days

Treatment group	No. of rats		Mean (\pm SEM) final body weight, g	Tumor incidence, % (% of control)*	Total no. of tumors in group*	Tumor multiplicity (% of control)*	Mean tumor weight, g (% of control)
	In group	With tumors					
Control	23†	15	287 \pm 4.6	65.2 (100)	33	1.43 (100)	2.79 (100)
Sulforaphane							
75 μ mol	20	7	272 \pm 5.1	35.0 (53.8)	9	0.45 (31.4)	1.24 (44.4)
150 μ mol	19‡	5	265 \pm 4.9	26.3 (40.3)	5	0.26 (18.2)	0.68 (24.4)
Compound 2							
75 μ mol	19§	4	268 \pm 5.1	21.0 (32.2)	5	0.26 (18.2)	1.10 (39.4)
150 μ mol	19§	4	274 \pm 7.0	21.0 (32.2)	5	0.26 (18.2)	1.12 (40.1)
Compound 3 (100 μ mol)	17¶	7	276 \pm 4.4	41.2 (63.3)	12	0.71 (49.7)	2.49 (89.2)
Compound 4 (100 μ mol)	20	8	286 \pm 4.8	40.0 (61.3)	8	0.40 (27.9)	1.98 (71.0)

*The results shown in these columns differ from those in Table 1 because only the 137 rats that survived to termination of experiment are analyzed.

†Two animals died with tumors before termination of the experiment and are not included.

‡One rat died immediately following gavage and is not included.

§One rat in each group died with tumors before termination of experiment and is not included.

¶Three rats died (two with tumors) before termination of the experiment and are not included.

respectively. The mean weight of the control tumors at termination of experiment was 2.79 g (Table 2).

Sulforaphane administered in five doses of either 75 or 150 μ mol blocked tumor development in a dose-dependent manner. At the higher dose of sulforaphane, the tumor incidence and multiplicity for all animals in the group were reduced to 38.7% and 16.7% of control values, respectively (Table 1). The magnitude of this protective effect was almost identical (40.3% and 18.2%, respectively) when only those animals surviving to the termination of the experiment were analyzed (Table 2). Sulforaphane also reduced the tumor weights to 44.4% and 24.4% of controls at the lower and higher doses, respectively (Table 2). Treatment with sulforaphane significantly delayed the development of tumors during the course of the experiment in comparison to the control group ($P =$

0.016 and 0.0022 at low and high doses of sulforaphane, respectively) (Fig. 1).

Norbornyl isothiocyanate 2 was an equally potent chemoprotector at the 75- and 150- μ mol doses, irrespective of whether incidence, multiplicity, tumor weight, or latency of tumor development was considered (Tables 1 and 2; Fig. 1). With these doses the protective effect appears to have attained a plateau. Since 75- μ mol doses of compound 2 reduced tumor incidence and multiplicity even more markedly than the same dose of sulforaphane, it is possible that 2 may be a more potent chemoprotector than sulforaphane.

Compounds 3 and 4, both tested at five doses of 100 μ mol, also blocked tumor formation, but these effects did not reach statistical significance for some indicators of protection (Tables 1 and 2). Thus, compound 3 was clearly the least potent

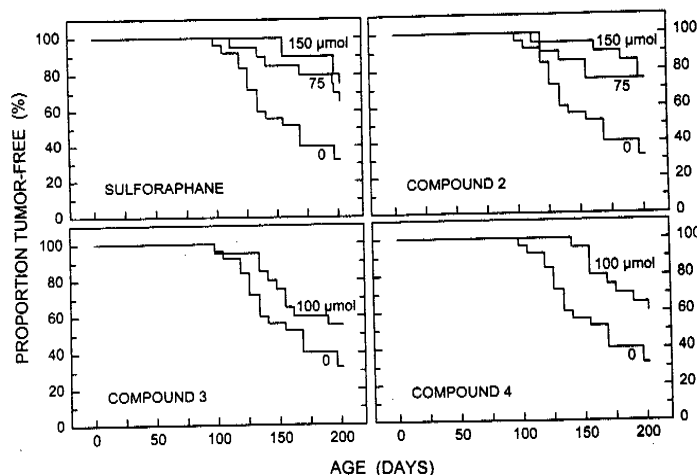


FIG. 1. Effect of treatment with sulforaphane (1) and norbornyl isothiocyanates (2-4) on mammary tumor development in female Sprague-Dawley rats that received 8 mg of DMBA at age 50 days. The proportion of tumor-free animals is shown at weekly intervals. The progression of tumor development in the control animals that received no protector (designated 0) is repeated in each panel. Sulforaphane: 75 μ mol ($P = 0.016$); 150 μ mol ($P = 0.0022$). Compound 2: 75 μ mol ($P = 0.023$); 150 μ mol ($P = 0.0074$). Compound 3: 100 μ mol ($P = 0.13$). Compound 4: 100 μ mol ($P = 0.023$). Kaplan-Meier incidence curves were analyzed by the logarithmic rank test. The P values refer to comparisons of differences in rate of tumor development in treated and control groups. All differences between treated and control groups are significant except for compound 3. The numbers of animals analyzed in each group are shown in Table 1.

protector. Although 3 produced some reduction in tumor incidence and multiplicity in comparison to controls, there was barely any reduction in tumor weights or significant prolongation in latency of tumor development. Compound 4 was apparently more potent than 3 but less potent than sulforaphane or compound 2 (Tables 1 and 2).

DISCUSSION

Sulforaphane, a naturally occurring isothiocyanate, and several structurally related synthetic 2-acetylnorbornyl isothiocyanates were tested for anticarcinogenic activity in this study, because they had previously been shown to be potent monofunctional inducers of phase 2 enzymes in cultured cells and in mouse tissues *in vivo* (5, 6). Since monofunctional induction has been proposed as a predictor of chemoprotective activity (7) and several structurally unrelated organic isothiocyanates have been shown to block chemical carcinogenesis and to induce Phase 2 enzymes (10, 11), we examined synthetic sulforaphane and the 2-acetylnorbornyl isothiocyanates 2–4 for chemoprotective activity. The present experiments demonstrate that all of these compounds, when administered around the time of carcinogen exposure, reduced—to varied degrees—the incidence, the multiplicity, and the weight of mammary tumors that developed in female Sprague–Dawley rats treated with DMBA. These agents also delayed tumor development. These observations further bolster the validity of the prediction that chemical compounds that induce phase 2 enzymes are promising candidates for achieving chemoprotection.

Sulforaphane was isolated as the principal and very potent phase 2 enzyme inducer from one variety of broccoli (5, 11). Our findings raise the issue to what extent sulforaphane and the many related inducers that are abundant in plants consumed by humans contribute to the chemoprotective activity of vegetables in man (12). There is insufficient information at present to draw conclusions on this matter.

Although our experiments do not provide a rigorous basis for comparing the relative potencies of sulforaphane (1) and compounds 2–4 as chemoprotectors, we can obtain some estimate of these potencies and relate them to the phase 2 enzyme inducer potencies (as measured by the concentrations required to double quinone reductase activities in murine hepatoma cells—i.e., the so-called CD values) (5, 6). These CD values are as follows: 0.2 μ M (sulforaphane); 0.3 μ M (compound 2); 0.8 μ M (compound 3); and 0.4 μ M (compound 4). A similar order of potency is reflected in the protective potencies of these compounds in blocking DMBA-induced mammary tumors. Compound 3 is the least potent protector. Sulforaphane and compound 2 are approximately equipotent, and 2 may be even more potent than sulforaphane. Sulforaphane and 2 are more potent than 3 or 4. It is therefore gratifying that measurements of inducer potencies in our cell culture assay not only correctly predicted anticarcinogenic activity but also provided a reasonable index of potency.

The reasons for the apparent differences in potencies of the compounds tested are not clear. The possibly higher potency of 2 in comparison to sulforaphane might be attributed to the

fact that the isothiocyanate group of 2 is secondary whereas that of sulforaphane is primary. Consequently, the former is likely to be less reactive and might therefore resist metabolic disposal or other promiscuous intracellular reactions with nucleophiles to which all isothiocyanates are susceptible. The differences in potencies of 2, 3, and 4 are more difficult to explain. In compounds 2 and 4 the functional groups are *exo*, whereas in compound 3 the 2-acetyl group is *endo* and, therefore, more protected. One aspect of this structure–activity relation is noteworthy: the nearly equivalent effects of methylsulfinyl ($\text{CH}_3\text{SO}-$) and methylcarbonyl ($\text{CH}_3\text{CO}-$) functions on both inducer and chemoprotective potencies of these agents.

The mechanisms of the chemoprotective actions of these compounds are not fully understood. Although isothiocyanates induce protective phase 2 enzymes, and the functionalized isothiocyanates used in these experiments are especially potent in this regard, it is becoming increasingly clear that some isothiocyanates also block activation of carcinogens by inhibiting phase 1 enzymes (10, 13). Whether sulforaphane and the 2-acetylnorbornyl isothiocyanates inhibit carcinogen activation is not known. Clearly, agents that are monofunctional inducers of phase 2 enzymes and block carcinogen activation by inhibiting phase 1 enzymes are likely to be ideal chemoprotectors.

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Regulatory Mechanisms of Monofunctional and Bifunctional Anticarcinogenic Enzyme Inducers in Murine Liver¹

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ABSTRACT

Anticarcinogenic enzyme inducers are of two types: (a) bifunctional inducers [2,3,7,8-tetrachlorodibenzo-*p*-dioxin, polycyclic aromatics, azo dyes, β -naphthoflavone] that elevate both Phase II enzymes [e.g., glutathione *S*-transferases, UDP-glucuronosyltransferases, and NAD(P)H:quinone-acceptor oxidoreductase] and certain Phase I enzymes [e.g., aryl hydrocarbon hydroxylase (AHH)]; and (b) monofunctional inducers [e.g., diphenols, thiocarbamates, 1,2-dithiol-3-thiones, isothiocyanates] that elevate primarily Phase II enzymes without significantly affecting AHH. Since Phase I enzymes such as AHH may activate precarcinogens to ultimate carcinogens whereas Phase II enzyme induction suffices to achieve chemoprotection, an understanding of the molecular mechanisms that regulate these enzymes is critical for devising methods for chemoprotection. We report a systematic analysis of the inductions of aryl hydrocarbon hydroxylase (AHH) and NAD(P)H:quinone reductase (QR) by seven monofunctional and eight bifunctional inducers, singly or in combination, in a murine hepatoma cell line (Hepa 1c1c7) and two mutants defective in either *Ah* (Aryl hydrocarbon) receptor function (BP^{c1}) or in AHH expression (c1). We have also examined such inductions in genetically defined mouse strains with high affinity (C57BL/6J) and low affinity (DBA/2J) *Ah* receptors. The combination of our earlier model for the induction of Phase I and Phase II enzymes (H. J. Prochaska, M. J. De Long, and P. Talalay, *Proc. Natl. Acad. Sci. USA*, 82: 8232, 1985) with mechanism(s) for autoregulation of AHH (O. Hankinson, R. D. Anderson, B. W. Birren, F. Sander, M. Nogishi, and D. W. Nebert, *J. Biol. Chem.*, 260: 1790, 1985) is compatible with our results. Thus, induction of QR by monofunctional inducers does not depend on a competent *Ah* receptor or AHH activity and appears to involve an electrophilic chemical signal. In contrast, bifunctional inducers require competent *Ah* receptors to induce both AHH and QR, although the latter process appears to be regulated by more than one mechanism. It is our view that bifunctional inducers bind to the *Ah* receptor thereby enhancing transcription of genes encoding both AHH and QR. Metabolizable bifunctional inducers are then converted by the induced AHH to products that resemble monofunctional inducers and are capable of generating the aforementioned chemical signal. The existence of mechanism(s) for AHH autoregulation that also affect Phase II enzyme expression would account for the high basal activities of QR in the AHH-defective mutant (c1).

INTRODUCTION

The protection of rodents against the toxic and neoplastic effects of chemical carcinogens can be achieved by a wide variety of seemingly unrelated chemical agents, including polycyclic aromatic hydrocarbons, azo dyes, flavonoids, phenolic antioxidants, isothiocyanates, diterpenes, indoles, unsaturated lactones, 1,2-dithiol-3-thiones, and thiocarbamates (1-4). Although a single mechanism cannot account for all forms of chemoprotection, it is clear that the induction of electrophile-

processing Phase II enzymes* (e.g., glutathione *S*-transferases, UDP-glucuronosyltransferases, and quinone reductase) is a major protective mechanism (3, 4, 6-11). Indeed, the induction of these enzymes is the single common biochemical effect shared by the aforementioned compounds, and monitoring of Phase II enzyme induction has permitted the isolation and identification of new anticarcinogens (1, 3, 4, 6-10).

Although all of the chemoprotectors described above are inducers of Phase II enzymes, large differences exist among these agents in their capacity to induce certain Phase I enzymes (3, 4). Thus, large planar aromatics such as polycyclic aromatic hydrocarbons, flavonoids, TCDD,⁵ and azo dyes enhance selected Phase I activities such as aryl hydrocarbon hydroxylase in rodents and in cultured murine hepatocytes (10, 12-18), yet minor and variable inductions of Phase I enzymes are evoked by the remaining classes of compounds (3, 4). Thus, these anticarcinogens can be segregated into bifunctional inducers that induce both AHH and Phase II enzymes and monofunctional inducers that selectively induce Phase II enzymes only (3, 4, 6, 9). These families of inducers have been designated as type A and type B by Wattenberg (3). Since Phase I enzyme induction is an important mechanism for the activation of many carcinogens to ultimate electrophiles (10, 19), and hence counteracts chemoprotection, whereas Phase II enzyme induction results in chemoprotection, an understanding of the mechanisms underlying these inductions is of critical importance for devising appropriate strategies for chemoprotection.

The molecular mechanism whereby bifunctional inducers (large planar aromatics) elevate AHH and related activities appears to be well established. These compounds bind avidly to the protein product of the *Ah* (Aryl hydrocarbon) locus, and ligand-bound receptors bind to enhancer regions of selected cytochrome P-450 genes, resulting in elevation of AHH activity and increased metabolism of aromatic hydrocarbons (12, 16, 18, 20-24). Because the induction of Phase II enzymes by planar aromatics generally occurs only in mouse strains and cultured hepatocytes with a functional *Ah* locus, the regulation of these enzymes has been assumed to occur through the same mechanism as that regulating AHH (25-27). Nevertheless, experiments attempting to demonstrate the direct participation of the *Ah* locus in the regulation of Phase II enzymes have not been convincing (28-30).

In contradistinction, monofunctional inducers have little apparent structural similarity. Their mechanism does not appear

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* The enzymes involved in the metabolism of xenobiotics have been classified into two broad categories (5). Phase I enzymes (which include the cytochromes P-450) functionalize compounds by oxidation, reduction, or hydrolysis, whereas Phase II enzymes carry out the conjugations of functionalized compounds with endogenous ligands (e.g., glucuronic acid, glutathione, amino acids, and sulfate). Although quinone reductase does not promote a synthetic function, it may be classified as a Phase II enzyme since it does not introduce new functional groups, is often induced coordinately with conjugation enzymes, and protects cells against the toxicities of quinones (6).

⁵ Abbreviations and trivial names used are: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; QR, NAD(P)H:quinone-acceptor oxidoreductase (EC 1.6.99.2) also known as DT-diaphorase or menadione reductase; AHH, aryl hydrocarbon hydroxylase (EC 1.14.14.1); *Ah*, Aryl hydrocarbon, the locus responsible for the induction by planar aromatics of aryl hydrocarbon hydroxylase (cytochrome P-450); Sudan III, 1-(4-phenylazophenylazo)-2-naphthol; oltipraz, 5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione.

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to involve a conventional receptor and does not require a functional *Ah* receptor (31, 32). Indeed, data presented previously (33) indicate that the inductive capacity of a wide range of phenolic antioxidants and phenylenediamines is critically dependent on the chemical properties of the inducers rather than their morphological features. Thus, in a murine hepatoma cell culture, a variety of hydroquinones (1,4-diphenols) and catechols (1,2-diphenols) are inducers of QR activity, whereas resorcinols (1,3-diphenols) are completely inactive. We have suggested that the observed inductions are the result of susceptibility to oxidations since catechols and hydroquinones have similar chemical properties in that they can undergo reversible one- or two-electron oxidations to the corresponding semiquinones or quinones, respectively. In contrast, resorcinols cannot undergo such facile oxidations. Hence, appropriate chemical reactivity rather than precise structure appears to be important for enzyme induction by diphenols. We have suggested that other classes of monofunctional inducers must possess electrophilic centers such as α,β -unsaturated carbonyl functions in order to exert inductive activity (34). Furthermore, we have proposed that the linkage of Phase II enzyme induction by bifunctional planar aromatics to *Ah* locus function may involve, at least in part, a metabolic cascade. Bifunctional inducers bind to the *Ah* receptor and thereby specify the enhanced synthesis of cytochrome P₁-450, which in turn converts bifunctional planar aromatics into species (e.g., 1,2- or 1,4-diphenols, diamines, araminophenols) that behave like monofunctional inducers (see Fig. 3).

Because of uncertainty over the relation between the regulation of the induction of these enzymes, we have undertaken a systematic analysis of this relationship with a variety of structurally dissimilar Phase II enzyme inducers in cultured cells and genetically defined mice. The induction of AHH activity, which is a measure of cytochrome P₁-450 levels, was used as an index of the induction of Phase I enzymes under the control of the *Ah* locus (12). QR activities were used as markers for Phase II enzyme induction. The majority of the experiments were done with the Hepa 1c1c7 murine hepatoma cell line in which the induction of both of these types of enzymes has been demonstrated (16-18, 20, 22-24, 31-33, 35-38). Induction experiments have also been carried out in two mutants of the Hepa 1c1c7 cell line that are defective in the *Ah* receptor or in AHH expression, as well as in mouse strains with high (C57BL/6J) and low (DBA/2J) affinity *Ah* receptors (12). These results have enabled the construction of a model for the mechanisms of induction of Phase I and Phase II enzymes.

MATERIALS AND METHODS

Materials. Flavin adenine dinucleotide, menadione, 2,6-dichlorodiphenol, bovine serum albumin, Tris base, Tween 20, 1-(2-pyridylazo)-2-naphthol, and 1-(2-thiazolylazo)-2-naphthol were obtained from Sigma (St. Louis, MO); 2,3,7,8-tetrachlorodibenzo-*p*-dioxin was from IIT Research Institute (Chicago, IL); NADH was from Pharmacia P-L Biochemicals (Piscataway, NJ); 3-hydroxybenzo(a)pyrene was from the Chemical Carcinogen Reference Standard Repository (National Cancer Institute, Bethesda, MD); 75-cm² culture plates were from Falcon (Becton Dickinson Labware, Oxnard, CA); α -minimal essential medium and fetal calf serum were from GIBCO (Grand Island, NY); dimethyl sulfoxide, hexane, acetonitrile, and ethyl acetate were from Burdick and Jackson (Muskegon, MI); Emulphor EL-620P was from GAF (Linden, NJ); sesame oil was from Fisher (Fair Lawn, NJ). Other inducing agents were obtained and prepared as described previously (31, 33, 37). Hepa 1c1c7 cells and their mutants were gifts of J. P. Whitlock, Jr., Stanford University, and O. Hankinson, University of California, Los Angeles.

Treatment of Hepa 1c1c7 Cells and Assay of Enzymatic Activities. Wild-type and mutant Hepa 1c1c7 cells were plated, grown, and induced as described (31-33, 37). After 24 h of exposure to inducing agents, the cells were washed with ice-cold 0.15 M KCl-10 mM potassium phosphate (pH 7.4), removed from the plates by scraping, and sonically disrupted for 5 s (Branson Sonifier Cell Disruptor 200). Two 200- μ l aliquots of the resulting 1.0- to 1.5-ml suspensions were assayed for AHH activity as described by Nebert (39). The remaining samples were centrifuged at 5000 \times g for 20 min and assayed for quinone reductase activity by measuring the rate of oxidation of NADH (200 μ M) by menadione (50 μ M) at 340 nm in the assay system described by Prochaska and Talalay (40). Protein concentrations were determined by the method of Bradford (41).

Treatment of Animals. Female C57BL/6J and DBA/2J mice (The Jackson Laboratory, Bar Harbor, ME), 5 weeks old, were housed in hanging stainless steel cages (5 mice/cage) without bedding at 24-25°C with light-dark cycles of 12 h each. The mice were fed powdered 5001 Purina laboratory chow (Ralston-Purina, St. Louis, MO). After the mice were allowed to acclimatize to their new environment for 2 weeks, they were either: (a) fed by gavage one of the following test compounds: 75 μ mol of *tert*-butylhydroquinone, 35 μ mol of 3,5-di-*tert*-butylcatechol, or 35 μ mol of 4,6-di-*tert*-butylresorcinol, in 0.1 ml of Emulphor; or (b) given i.p. injections of test compound (5 μ mol of β -naphthoflavone, Sudan III, or 1-(2-thiazolylazo)-2-naphthol) in 0.2 ml of sesame oil. Both types of treatments were given daily for 5 days. Control groups received the respective vehicles only by the same routes. Mice were killed by cervical dislocation 24 h after the last dose and the livers were excised, perfused with 0.15 M KCl-2 mM EDTA (pH 7.4), frozen immediately in liquid nitrogen, and stored at -80°C until used.

Preparation of Mouse Liver Subcellular Fractions and Assay of Their Enzymatic Activities. Portions (500 mg) from each liver were homogenized in 1.5 ml of 0.15 M KCl, 10 mM potassium phosphate, and 0.5 mM EDTA, pH 7.4 ("homogenization buffer"). After centrifugation at 10,000 \times g for 30 min, the postmitochondrial supernatant fractions were centrifuged at 90,000 \times g for 75 min. The resulting cytosols were collected and assayed for: (a) QR by following the reduction of 2,6-dichlorodiphenol (40 μ M) by NADH (200 μ M) at 600 nm (40); (b) glutathione S-transferase with glutathione and 1-chloro-2,4-dinitrobenzene as substrates according to the procedure of Habig *et al.* (42); and (c) protein content by the method of Bradford (41). Microsomal pellets were suspended in 5 ml of homogenization buffer and centrifuged at 90,000 \times g for 90 min, resuspended in 0.5 ml of homogenization buffer, frozen in liquid nitrogen, and stored at -80°C. The microsomal fractions were subsequently assayed for: (a) AHH activity with benzo(a)pyrene and NADPH as substrates according to the method of Nebert (39); (b) P-450 levels according to the method of Omura and Sato (43); and (c) protein content according to the method of Bradford (41).

Statistical Treatment of Results. The results in the figures and Tables 1-3 are displayed as the ratios of the specific activities (or levels) of treated samples to those of controls. The standard error for each ratio was divided by the appropriate control value. The means \pm SE for the control groups are given in Tables 1 and 3 and in the appropriate figure legends. All results shown were obtained from at least duplicate measurements of four individual animals or four culture plates per treatment group.

RESULTS

In the studies described below we measured the specific activities of cytosolic QR and AHH in Hepa 1c1c7 cells and two mutants with defective AHH expression, as well as in livers of two inbred mouse strains, one of which (C57BL/6J) is responsive to induction of AHH by polycyclic hydrocarbons, while the other (DBA/2J) is unresponsive (12). To analyze the mechanisms of regulation of the induction of these enzymes by various chemoprotective agents we examined: (a) the relation of structure of the agents to their ability to induce the two types of activities; (b) dose-response relationships; and (c) the effects

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of combinations of maximally responsive concentrations of various types of inducers.

Induction of Quinone Reductase and Aryl Hydrocarbon Hydroxylase by Two Classes of Chemoprotectors. Fig. 1 provides a direct comparison of the induction in Hepa 1c1c7 murine hepatoma cells of QR (top) and AHH (bottom) by 15 compounds that are known to be inducers of these enzymes in rodent tissues (6). All of the compounds are recognized protectors against the neoplastic or toxic effects of chemical carcinogens (1-4, 6-9, 44, 45) under appropriate experimental conditions, although several are also carcinogens. In this experiment the hepatoma cells were grown to near confluence and then exposed for 24 h to concentrations of the inducers (10 nM to 30 μ M) selected to produce moderate to maximal inductions without causing obvious cytotoxicity. The results provide a striking and nearly absolute distinction between two types of inducers: (a) bifunctional inducers which are exemplified by polycyclic aromatics, β -naphthoflavone, azo dyes, and TCDD; and (b) monofunctional inducers which include *tert*-butylhydroquinone, 3,5-di-*tert*-butylcatechol, bisethoxyanthogen, 1,2-dithiol-3-thione, oltipraz, and benzylisothiocyanate. Whereas bifunctional inducers elevated both QR (2 to 4 times control levels) and AHH (10 to 30 times control levels), monofunctional inducers had no significant effect on AHH while inducing QR to values similar to those observed with bifunctional inducers.

One other important observation (Fig. 1) was that under identical conditions, the relative induction of the two enzyme activities by different bifunctional inducers varied considerably. For instance, at equimolar concentrations (2 μ M), benzo-

(a)pyrene and 3-methylcholanthrene were more effective than was Sudan III in inducing QR, whereas Sudan III produced a much greater induction of AHH than did the two polycyclic hydrocarbons. Furthermore, 1-(2-pyridylazo)-2-naphthol behaved anomalously from the other azo dyes in that it was a relatively poor inducer of AHH (about 2-fold) while raising the level of QR 3.5-fold. Since the ratios of induction of QR to AHH vary greatly from 0.0775 for Sudan III to 1.58 for 1-(2-pyridylazo)-2-naphthol, it seems evident that bifunctional inducers regulate QR and AHH by different mechanisms. Further evidence for several mechanism of induction will be developed below on the basis of dose-response curves, results obtained in mice *in vivo*, and the simultaneous use of saturating doses of both types of inducers.

Dose-Response Relationships in the Induction of Quinone Reductase and Aryl Hydrocarbon Hydroxylase by Monofunctional and Bifunctional Inducers. Because the degree of induction (ratio of treated to control specific activities) of AHH and QR by various inducers was not constant (Fig. 1), we examined the dose responses of these enzymes to selected compounds in the murine hepatoma Hepa 1c1c7 cell line (Fig. 2). The dose-response curves of induction of QR and AHH by TCDD were similar, suggesting a common mechanism of induction. TCDD was clearly the most potent inducer of both activities. TCDD was also the most effective inducer of AHH, yet it was not the most effective inducer of QR. Furthermore, differences in the regulation of AHH and QR by bifunctional inducers are apparent since the dose responses to Sudan III, 1,1'-azobenzene, and 1-(2-pyridylazo)-2-naphthol for these enzymes are quite different. Lastly, 1,2-dithiol-3-thione was completely inactive as an inducer of AHH yet it induced QR about as effectively as did Sudan III or TCDD.

Induction of Quinone Reductase and Aryl Hydrocarbon Hydroxylase by Combinations of Saturating Concentrations of Monofunctional and Bifunctional Inducers. Fig. 2 shows that concentrations of TCDD higher than 200 pM and of 1,2-dithiol-3-thione greater than 10 μ M produced maximal inductions of QR in Hepa 1c1c7 cells. The exposure of these cells to saturating concentrations of both compounds simultaneously produced

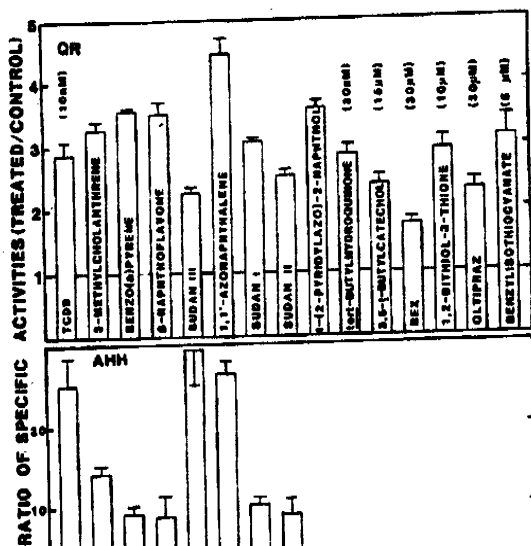


Fig. 1. Structure-activity study identifying monofunctional and bifunctional inducers. The induction profiles for QR and AHH in Hepa 1c1c7 murine hepatoma cells by 15 inducers of QR were determined and are expressed as specific activity ratios of treated to control cells. The concentrations of inducers were 2 μ M unless otherwise specified. Note that among polycyclic aromatics, the relationships between QR and AHH specific activities are not constant. The specific activities for QR and AHH in control cells were 297 ± 15 nmol/min/mg and 1.40 ± 0.14 pmol/min/mg, respectively. BEZ, bisethoxyanthogen; Sudan I, 1-(phenylazo)-2-naphthol; Sudan II, 1-(2,4-dimethylphenylazo)-2-naphthol.

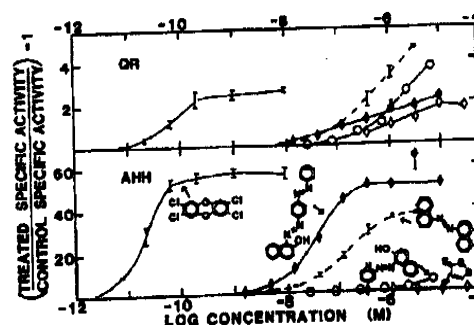


Fig. 2. Concentration dependence of induction of QR and AHH by TCDD, Sudan III, 1,1'-azobenzene, 1-(2-pyridylazo)-2-naphthol, and 1,2-dithiol-3-thione. The specific activities of QR and AHH in Hepa 1c1c7 murine hepatoma cells were determined as a function of concentrations and are expressed as ratios of treated to control cells (minus 1). TCDD is clearly the most potent inducer of both activities. TCDD is also the most effective inducer of AHH, yet it is not the most effective inducer of QR. 1,2-Dithiol-3-thione has no effect on AHH and the azo dyes tested show a variety of dose-concentration responses. These data suggest that multiple mechanisms for the induction of QR exist. The control values for QR and AHH were 349 ± 18 nmol/min/mg and 1.22 ± 0.08 pmol/min/mg, respectively.

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more than additive inductions of QR (Table 1). Thus 1–10 nM TCDD and 10–30 μ M 1,2-dithiol-3-thione elevated QR to 3.8 and 2.6 times control levels, respectively, whereas the combination produced more than an 8-fold induction. In contrast, the induction of AHH in Hepa 1c1c7 cells by saturating concentrations of TCDD (10 nM) was not affected by 30 μ M 1,2-dithiol-3-thione (Table 1) which by itself had a minimal effect on AHH activity (Figs. 1 and 2, Table 1).

Role of Ah Receptor Function in the Induction of Phase II Enzymes. Although the participation of the Ah receptor in the induction of Phase II enzymes by bifunctional inducers has been shown in inbred mice (25, 28, 29), such an association has not been clearly demonstrated with monofunctional inducers *in vivo*. We found that in livers of such inbred mouse strains (Table 2) monofunctional inducers acted independently of the Ah receptor since *tert*-butylhydroquinone and 3,5-di-*tert*-butylcatechol (but not 4,6-di-*tert*-butylresorcinol) induced glutathione *S*-transferase and QR in DBA/2J mice which have low affinity (defective) Ah receptors. The findings that 1,2- and 1,4-diphenols but not a 1,3-diphenol are inducers of QR in mice are

completely compatible with those reported with cell cultures (31–33). Furthermore, although planar aromatics such as Sudan III and β -naphthoflavone induced QR, glutathione *S*-transferase, and AHH activities in C57BL/6J but not in DBA/2J mice, the finding that 1-(2-thiazolylazo)-2-naphthol could induce Phase II enzymes in both strains of mice without greatly influencing AHH activity demonstrates that some bifunctional inducers may elevate Phase II enzymes in large part independently of the Ah receptor. Similar structure-activity relationships have been reported in rat liver and Hepa 1c1c7 cells (14, 31, 32).

To assess the role of the Ah receptor in the greater than additive induction phenomenon described above, the effects of combining saturating doses of monofunctional (1,2-dithiol-3-thione) with bifunctional (TCDD or β -naphthoflavone) inducers were examined in Hepa 1c1c7 cells as well as in its mutants defective in either a functional Ah receptor (BP^c1) (31, 32, 46) or the cytochrome P₄₅₀ gene (c1) (18, 38, 47). The BP^c1 and c1 mutants have low and high basal specific activities of QR, respectively (Table 3; Refs. 31, 32, and 38). Neither mutant has detectable AHH activity. Table 3 shows that the combination of 1,2-dithiol-3-thione with either TCDD or β -naphthoflavone at saturating concentrations resulted in augmented induction of QR in Hepa 1c1c7 (wild-type) cells. Furthermore, an additive elevation of QR was observed when TCDD was combined with β -naphthoflavone. This increase in QR induction by addition of β -naphthoflavone to concentrations of TCDD that saturate all the Ah receptors argues that β -naphthoflavone induces QR via an alternate pathway. No combination of compounds gave AHH activities that were higher than those obtained with TCDD alone (not shown). In cells with defective Ah receptor function (BP^c1) or in mutants that produce a defective AHH gene transcript (c1), 1,2-dithiol-3-thione was the only compound tested that was capable of inducing QR effectively, although slight elevations of QR by bifunctional inducers were noted in the c1 mutant. The addition of bifunctional inducers to 1,2-dithiol-3-thione had no effect on the induction of QR in the BP^c1 mutant although marginal increases were found with the c1 mutant. Hence monofunctional inducers act independently of the Ah receptor, whereas bifunctional inducers require

Table 1 Induction of quinone reductase and aryl hydrocarbon hydroxylase in Hepa 1c1c7 murine hepatoma cells by maximally inducing concentrations of TCDD and 1,2-dithiol-3-thione, singly and in combination

The results are expressed as ratios of specific activities of inducer-treated cells to controls.

Inducers	Concentration	Ratio of specific activities (treated/control)	
		Quinone reductase	Aryl hydrocarbon hydroxylase
TCDD	1 nM	3.66 \pm 0.09 ^a	30.4 \pm 1.8
	10 nM	3.92 \pm 0.03	36.8 \pm 5.6
1,2-Dithiol-3-thione	10 μ M	2.57 \pm 0.19	1.17 \pm 0.15
	30 μ M	2.67 \pm 0.10	1.29 \pm 0.05
TCDD and 1,2-dithiol-3-thione	10 nM	8.53 \pm 0.07 ^b	27.7 \pm 2.5
	30 μ M		

^a The standard error for each entry has been divided by the control value.

^b If the effects of TCDD and 1,2-dithiol-3-thione on QR were strictly additive a ratio of 5.59 would have been expected.

^c The mean control values for QR and AHH were (\pm SE) 230 \pm 5 nmol/min/mg and 0.959 \pm 0.069 pmol/min/mg, respectively.

Table 2 Induction patterns of hepatic quinone reductase, glutathione *S*-transferase, aryl hydrocarbon hydroxylase, and cytochrome P-450 levels in inbred Ah (aryl hydrocarbon) receptor-positive (C57BL/6J) and -negative (DBA/2J) mice

The results are expressed as ratios of specific activities (or levels) of treated to control livers. The means \pm SE were determined from four livers.

Inducer	Dose/day (μ mol)	Ratio of treated to control							
		C57BL/6J mice				DBA/2J mice			
		Quinone reductase	Glutathione <i>S</i> -transferase	Aryl hydrocarbon hydroxylase	Cytochrome P-450 levels	Quinone reductase	Glutathione <i>S</i> -transferase	Aryl hydrocarbon hydroxylase	Cytochrome P-450 levels
Sudan III	5	2.19 \pm 0.19	1.45 \pm 0.10	8.24 \pm 0.96	1.49 \pm 0.15	0.88 \pm 0.04	0.92 \pm 0.05	0.96 \pm 0.18	0.82 \pm 0.06
β -Naphthoflavone	5	3.59 \pm 0.26	2.15 \pm 0.05	6.70 \pm 0.44	1.56 \pm 0.17	1.24 \pm 0.05	1.29 \pm 0.05	0.96 \pm 0.26	0.93 \pm 0.11
1-(2-Thiazolylazo)-2-naphthol	5	4.17 \pm 0.18	2.56 \pm 0.11	2.20 \pm 0.41	0.85 \pm 0.10	1.94 \pm 0.16	1.86 \pm 0.15	1.05 \pm 0.14	0.87 \pm 0.05
3,5-di- <i>tert</i> -Butylcatechol	35	— ^a	— ^a	— ^a	— ^a	3.83 \pm 0.32	4.74 \pm 0.34	0.69 \pm 0.07	0.83 \pm 0.14
4,6-di- <i>tert</i> -Butylresorcinol	35	— ^a	— ^a	— ^a	— ^a	1.21 \pm 0.18	1.13 \pm 0.14	1.01 \pm 0.14	0.93 \pm 0.10
<i>tert</i> -Butylhydroquinone	75	2.12 \pm 0.08	2.15 \pm 0.13	1.23 \pm 0.11	1.22 \pm 0.14	2.37 \pm 0.13	2.65 \pm 0.24	0.98 \pm 0.18	0.73 \pm 0.13

Control values for mouse livers:

Mouse	Treatment	Glutathione <i>S</i> -transferase (nmol/min/mg)	Quinone reductase (nmol/min/mg)	Aryl hydrocarbon hydroxylase (pmol/min/mg)	Cytochrome P-450 levels (pmol/mg)
C57BL/6J	Sesame oil	2470 \pm 40 ^b	203 \pm 7	76.9 \pm 9.8	583 \pm 54
	Emulphor	2950 \pm 380	218 \pm 29	80.4 \pm 7.2	415 \pm 30
DBA/2J	Sesame oil	1890 \pm 100	160 \pm 16	84.6 \pm 4.5	437 \pm 45
	Emulphor	2190 \pm 240	144 \pm 14	74.9 \pm 10.2	491 \pm 35

^a Significant toxicity. Livers were not assayed.

^b Mean \pm SE of four livers.

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Table 3 Effect of combining bifunctional inducers with monofunctional inducers on the quinone reductase activity of Hepa 1c1c7 (wild-type), BP^c1 (defective translocation of Ah receptor-ligand complex into nucleus), and c1 (defective cytochrome P₁-450 gene) cell lines

Cells were grown, induced, and assayed as described under "Materials and Methods." The treated/control ratios shown are the ranges for the means of two separate experiments. For each condition, three plates were assayed in one experiment and four in the other. Standard errors were less than 5% of the mean values. Note that aryl hydrocarbon hydroxylase was assayed and was found to be undetectable in BP^c1 and c1 mutants, while the degree of induction of aryl hydrocarbon hydroxylase in Hepa 1c1c7 cells is consistent with the results shown in Figs. 1 and 2 (i.e., no compounds or combination of compounds was more effective than TCDD alone). Control quinone reductase specific activities (\pm SEM) in the two experiments, respectively, were: Hepa 1c1c7, 333 ± 5 and 223 ± 10 ; BP^c1, 164 ± 3 and 113 ± 6 ; c1, 901 ± 20 and 937 ± 69 nmol/min/mg protein.

Inducers(s)	Concentration of inducers	Ratio of quinone reductase specific activities (treated/control)		
		Hepa 1c1c7	BP ^c 1	c1
1,2-Dithiol-3-thione	30 μ M	3.17-3.21	2.76-2.76	2.44-3.12
TCDD	10 nM	3.83-4.10	0.90-1.00	1.33-1.66
β -Naphthoflavone	5 μ M	5.13-6.07	0.87-0.98	1.20-1.36
1,2-Dithiol-3-thione + TCDD	30 μ M + 10 nM	9.53-9.59	2.60-2.66	3.21-4.53
1,2-Dithiol-3-thione + β -naphthoflavone	30 μ M + 5 μ M	7.08-7.63	2.53-2.71	2.42-4.71
TCDD + β -naphthoflavone	10 nM + 5 μ M	7.23-8.08	0.84-1.08	1.11-1.53

competent Ah receptors (also see Ref. 31 and 32). Furthermore, the role of AHH in the regulation of QR can be inferred from the results obtained with the c1 mutant since its genetic defect lies in the P₁-450 structural gene rather than the Ah receptor (47).

DISCUSSION

A model for the regulation of Phase II enzymes by monofunctional and bifunctional inducers proposed by us in 1985 (33) comprises three mechanisms (Fig. 3): In Mechanism A, monofunctional inducers activate the synthesis of Phase II enzyme by means of an electrophilic signal which operates independently of Ah receptors or the induction of AHH; in Mechanism B, complexes resulting from the combination of bifunctional inducers with Ah receptors bind to specific regions of nuclear DNA and thereby evoke enhanced transcription of both AHH and Phase II enzymes; and in Mechanism C, bi-

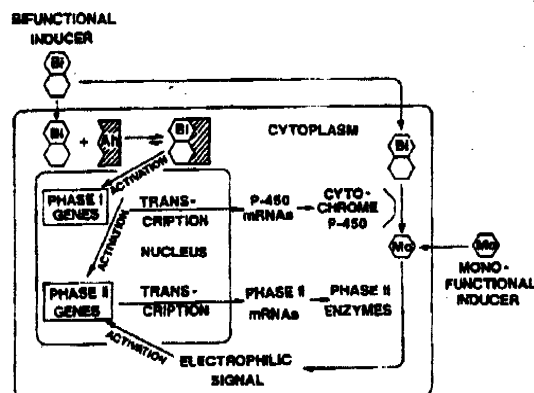


Fig. 3. Metabolic cascade model for the relation between the mechanism of action of monofunctional (Mo) and bifunctional (Bi) inducers of Phase I and Phase II enzymes [slightly modified from data of Prochaska *et al.* (33)]. Monofunctional inducers enter the cell and generate the electrophilic signal that stimulates the induction of Phase II enzymes only. Bifunctional inducers require participation of the Ah receptor in two distinct mechanisms of induction. Bifunctional inducers enter the cell and bind to the Ah receptor, and the resultant complex activates gene transcription for both Phase I and Phase II enzymes. The resulting enhanced AHH activity converts metabolizable bifunctional inducers into compounds analogous in electrophilic properties to monofunctional inducers, which signal Phase II gene transcription. This model and mechanism(s) for AHH autoregulation that also affect Phase II enzyme expression (18, 38) can reconcile the experimental findings.

functional inducers susceptible to metabolism by AHH (induced by Mechanism B) are converted to electrophilic products that elevate Phase II enzymes by Mechanism A.

This model accounts for the observations obtained with Hepa 1c1c7 murine hepatoma cells. Thus, we have identified two families of Phase II enzyme inducers which are differentiated by their ability to induce AHH and their dependence (or independence) on Ah receptors for their mechanism of action. Nonmetabolizable bifunctional inducers such as TCDD (48) act via the Ah receptor directly (Mechanism B) to induce AHH and Phase II enzymes, while metabolizable inducers act also via Mechanisms C and A, whereby the induced AHH converts these compounds to metabolites resembling monofunctional inducers. This formulation would account for the similar dose-response curves of induction of QR and AHH by TCDD, the dissimilarity of these curves for metabolizable bifunctional inducers, and the disparity in the degrees of induction of AHH and QR evoked by various bifunctional inducers. Furthermore, the operation of two different yet interactive mechanisms (B and C/A) of induction on Phase II enzymes would be in agreement with the more than additive inductions observed by the use of combination of saturating concentrations of monofunctional and bifunctional inducers. The proposal that metabolizable bifunctional inducers act via more than one mechanism is also supported by the observation that saturating concentrations of TCDD and β -naphthoflavone produce virtually additive inductions of QR.

Most of the observations obtained with AHH-defective mutant cells are also consistent with the model shown in Fig. 3. Thus, monofunctional inducers elevate QR to the same degree in mutant as in wild-type cells, including the c1 mutant which has high basal QR activity. Bifunctional inducers are completely inactive in the BP^c1 cell line since these cells have defective Ah receptors. This prevents bifunctional inducers from acting directly via Ah receptors (Mechanism B) as well as participating in a metabolic cascade (Mechanism C/A) since no AHH induction occurs. Surprisingly, bifunctional inducers were only weak inducers of QR in the c1 mutant. This was an unexpected result since this mutant has intact Ah receptors [the c1 mutant has its genetic defect in the cytochrome P₁-450 gene (47)]. Thus, bifunctional inducers should be able to induce QR by binding Ah receptors and directly activating genes coding for Phase II enzymes (Mechanism B).

In addition to the resistance of the c1 mutant to induction of QR by bifunctional inducers, there are other experimental findings with Hepa 1c1c7 cell mutants that cannot be accommodated by the above model. Hankinson *et al.* (18) observed that mutants with defects in their cytochrome P₁-450 gene had

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high levels of P₁-450 mRNA which could not be elevated further by TCDD. These high P₁-450 mRNA levels were suppressed by coculture or fusion with wild-type cells. Two models were proposed to account for the regulation of cytochrome P₁-450 and the high basal activities of Phase II enzymes which are refractory to further elevations by TCDD (18, 38). In the first model, an endogenous ligand for the Ah receptor that is susceptible to inactivation by AHH is postulated. In cells without AHH, but which possess functional Ah receptors, the ligand accumulates, binds to Ah receptors, and activates the transcriptional activity of AHH and Phase II enzymes. In wild-type cells, the ligand cannot accumulate since it is rapidly inactivated by AHH. The second model proposes that prerepressors exist which are activated by AHH. The active repressors then decrease the transcriptional activity for AHH and Phase II enzymes. Hence, cells defective in AHH would not be able to convert prerepressors to active repressors that result in the high constitutive expression of AHH and Phase II enzymes.

We conclude that the model shown in Fig. 3 together with the postulated existence of mechanism(s) for the autoregulation of AHH that have similar effects on Phase II enzyme expression can account for the induction patterns of monofunctional and bifunctional inducers (alone or in combination), as well as the profiles of QR which Hepa 1c1c7 mutants exhibit.

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Direct Measurement of NAD(P)H:Quinone Reductase from Cells Cultured in Microtiter Wells: A Screening Assay for Anticarcinogenic Enzyme Inducers

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We describe a rapid and direct assay of NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2) activity in cultured cells suitable for identifying and purifying inducers of this detoxication enzyme. Hepa 1c1c7 murine hepatoma cells are plated in 96-well microtiter plates, grown for 24 h, and exposed to inducing agents for another 24 h. The cells are then lysed and quinone reductase activity is assayed by the addition of a reaction mixture containing an NADPH-generating system, menadione (2-methyl-1,4-naphthoquinone), and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Quinone reductase catalyzes the reduction of menadione to menadiol by NADPH, and MTT is reduced nonenzymatically by menadiol resulting in the formation of a blue color which can be quantitated on a microtiter plate absorbance reader. The reaction is more than 90% dicoumarol-inhibitable and menadione dependent. The results are comparable to those obtained by harvesting cells from larger plates, preparing cytosols, and carrying out spectrophotometric measurements. © 1988 Academic Press, Inc.

KEY WORDS: quinone reductase; phase II enzymes; enzyme induction; microtiter plates; anticarcinogens.

We have developed a rapid, efficient, and inexpensive assay for measuring NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2) from cells cultured in microtiter wells. Quinone reductase² is a widely distributed, primarily cytosolic, dicoumarol-inhibitable flavoprotein that catalyzes the reduction of a wide variety of quinones and quinoneimines (1,2). Quinone reductase protects cells against the toxicity of quinones and their metabolic precursors by promoting the obligatory two-electron reduction of quinones to hydroquinones which are then sus-

ceptible to glucuronidation (3-10). In addition, quinone reductase is induced coordinately with other electrophile-processing Phase II enzymes (glutathione *S*-transferases and UDP-glucuronosyltransferases) by a variety of compounds that protect rodents from the toxic, mutagenic, and neoplastic effects of carcinogens (2,11-13). There is a large body of evidence which suggests that the induction of Phase II enzymes is the predominant mechanism by which these heterogeneous compounds are chemoprotective (11-15), and it is clear that the monitoring of Phase II enzyme induction is a convenient method for screening for anticarcinogenic activity (11-13,15-23).

Although many anticarcinogenic enzyme inducers have been discovered, other unrecognized compounds may exist that are potent, effective, and nontoxic (e.g., the active constituents from poorly characterized plant

extracts) (11,17,20-screening compounds induce Phase II enzyme time-consuming, and laboratory has recent rapid screening system that quinone reductase Hepa 1c1c7 murine many of the compounds *in vivo* (24-conventional assay technique homogenizing, centrifugation, enzymatic activity at time-consuming and fullness of this procedure have developed a direct assay for quinone reductase from cells grown in well microtiter plates. NADPH-dependent reduction of MTT. This is a rapid, accurate, inexpensive screening method for many compounds of concentrations of experiment, and automated data processing. This assay is important for chemoprevention and medicinal interest.

EXPERIMENTAL

Materials

MTT, NADP, FAD, serum albumin, Tris, phosphate, bakers' yeast, hydrogenase, Tween 20, mycophenolate, and crystalline NADH were from Sigma Chemical Co. (St. Louis, MO). NADH was from Pica Chemicals (Piscataway, NJ). Fetal calf serum was from Falcon (Becton Dickinson, Oxnard, CA); α -minoxidipril was from Grand Island, NY). Nitrile were from Muskegon, MI). Benzo-*p*-dioxin (TC-119) was from IIT Research Institute.

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² Abbreviations used: quinone reductase, NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2); DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sudan I, 1-phenylazo-2-naphthol; Sudan II, 1-(2,4-dimethylphenylazo)-2-naphthol; Sudan III, 1-(4-phenylazophenylazo)-2-naphthol.

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extracts) (11,17,20-23). Unfortunately, screening compounds for their ability to induce Phase II enzymes in animals is difficult, time-consuming, and expensive (17,20). Our laboratory has recently developed a more rapid screening system by demonstrating that quinone reductase is induced in the Hepa 1c1c7 murine hepatoma cell line by many of the compounds that induce Phase II enzymes *in vivo* (24-26). Nevertheless, conventional assay techniques (e.g., harvesting, homogenizing, centrifuging, and assaying for enzymatic activity and protein content) are time-consuming and therefore limit the usefulness of this procedure. For this reason, we have developed a direct assay of quinone reductase from cells grown and induced in 96-well microtiter plates by measuring the NADPH-dependent menadiol-mediated reduction of MTT. This assay procedure is rapid, accurate, inexpensive, capable of screening many compounds and/or a series of concentrations of compounds in a single experiment, and amenable to computerized data processing. This method should facilitate the identification of new and potentially important chemoprotective compounds of medicinal interest.

EXPERIMENTAL PROCEDURES

Materials

MTT, NADP, FAD, menadione, bovine serum albumin, Tris base, glucose 6-phosphate, bakers' yeast glucose-6-phosphate dehydrogenase, Tween-20, penicillin G, streptomycin, and crystal violet were obtained from Sigma Chemical Co. (St. Louis, MO); NADH was from Pharmacia P-L Biochemicals (Piscataway, NJ); microtiter wells were from Falcon (Becton-Dickinson Labware, Oxnard, CA); α -minimal essential medium and fetal calf serum were from GIBCO (Grand Island, NY); and DMSO and acetonitrile were from Burdick and Jackson (Muskegon, MI). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained from IIT Research Institute (Chicago, IL). Other

inducing agents were obtained and prepared as described previously (24-26). Hepa 1c1c7 cells were a gift of J. P. Whitlock, Jr., Stanford University. Multiple pipettors (50- and 200- μ l Octapipets) were purchased from Costar (Cambridge, MA). The absorbances of microtiter wells were measured with an automated optical scanner equipped with a 610-nm filter (Biotek, Winooski, VT).

Methods

Growth of cells. Hepa 1c1c7 cells were plated at a density of 10 000 cells/well in 200 μ l of α -minimal essential medium (without ribonucleosides or deoxyribonucleosides) supplemented with 10% fetal calf serum. The cells were grown for 24 h in a humidified incubator in 5% CO₂ at 37°C. The medium was decanted and each well was refed with 200 μ l of α -minimal essential medium supplemented with 10% fetal calf serum, 100 U/ml of penicillin G, 100 μ g/ml of streptomycin, and 0.1% DMSO. Compounds to be tested as inducers were dissolved in DMSO and were diluted into the media so that the final concentration of DMSO was 0.1% by volume. Control cells were always grown in the second column of wells and were fed media containing 0.1% DMSO. The cells were then incubated for an additional 24 h.

Assay of quinone reductase. The following stock solution was prepared for each set of assays: 7.5 ml of 0.5 M Tris-Cl (pH 7.4), 100 mg of bovine serum albumin, 1 ml of 1.5% Tween-20, 0.1 ml of 7.5 mM FAD, 1 ml of 150 mM glucose 6-phosphate, 90 μ l of 50 mM NADP, 300 U of yeast glucose-6-phosphate dehydrogenase, 45 mg of MTT, and distilled water to a final volume of 150 ml. Menadione (1 μ l of 50 mM menadione dissolved in acetonitrile per milliliter of reaction mixture) was added just before the mixture was dispensed into the microtiter plates.

After the plates were exposed to test compounds for 24 h, the media were decanted, and the cells were lysed by incubation at 37°C for 10 min with 50 μ l in each well of a solution containing 0.8% digitonin and 2

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mm EDTA, pH 7.8. The plates were then agitated on an orbital shaker (100 rpm) for an additional 10 min at 25°C, after which 200 μ l of the complete reaction mixture was added to each well with the aid of a multiple pipetting device (Octapipet). A blue color developed and the reaction was arrested after 5 min by the addition of 50 μ l of a solution containing 0.3 mM dicoumarol in 0.5% DMSO and 5 mM potassium phosphate, pH 7.4. The plates were then scanned at 610 nm. The first column of wells in the plates always contained the reaction mixture only and served as the nonenzymatic blank. The average absorbance value of this column of wells was subtracted automatically from all other absorbance readings.

In order to determine the proportion of MTT reduction attributable to quinone reductase activity (Table 1), three sets of microtiter plates were grown and induced under identical conditions. The cells on one set of plates were assayed as described above. A second set of cell lysates was assayed in the presence of 50 μ l per well of 0.3 mM dicoumarol in 0.5% DMSO and 5 mM potassium phosphate (pH 7.4). The third set of cells was lysed in the standard fashion but assayed with a reaction mixture containing no men-

adione. The absorbances were scanned 5 min after the addition of the reaction mixture.

Crystal violet staining. Since some quinone reductase inducers or crude fractions that are being screened for inducer activity depress the rate of cell growth, it is desirable to relate the observed quinone reductase activity to the number of cells or the amount of protein in each microtiter well. This normalization can be conveniently accomplished by staining a set of microtiter plates treated identically to those used for the MTT assay with crystal violet. We have used a slight modification of the method of Drysdale *et al.* (27) for this purpose. The media were decanted, the plates were submerged in a vat of 0.2% crystal violet in 2% ethanol for 10 min and rinsed for 2 min with tap water, and the bound dye was solubilized by incubation at 37°C for 1 h with 200 μ l of 0.5% sodium dodecyl sulfate in 50% ethanol. The plates were then scanned at 610 nm.

In order to demonstrate the validity of the crystal violet assay as a convenient measure of protein content and cell number, five twofold serial dilutions of Hepa 1c1c7 cells were plated in each of six identical 24-well 2-cm² plates (four wells per dilution of cells). The cells were grown for 24 h, refed with

COMPARISON OF QUINONE GROWN IN MICROTITER WELLS

Compounds

Polycyclic aromatics
2,3,7,8-Tetrachlorodibenzo-
 β -Naphthoflavone
Benzo[*a*]pyrene
3-Methylcholanthrene

Azo dyes

1,1'-Azonaphthalene
1-(2-Pyridylazo)-2-naphth-
1-(2-Thiazolylazo)-2-naph-
Sudan I
Sudan II
Sudan III

Diphenols

Catechol
Resorcinol
Hydroquinone
tert-Butylcatechol
tert-Butylresorcinol
tert-Butylhydroquinone

Isothiocyanate

Benzylisothiocyanate

Dithiolthiones

1,2-Dithiol-3-thione
4-Phenyl-1,2-dithiol-3-th-
5-(2-Pyrazinyl)-4-methyl
dithiol-3-thione

- * Direct assay described
- * Hepa 1c1c7 cells grown
- * Mean value \pm standard
- * Unpublished results (N)
- * From Prochaska *et al.*

medium, and grown. The total cellular pro-
plate was determined
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TABLE 1

RATES OF MTT REDUCTION OF CONTROL, β -NAPHTHOFLAVONE OR 1,2-DITHIOL-3-THIONE-TREATED
HEPA 1c1c7 CELLS GROWN IN MICROTITER WELLS

Treatment of cells	Number of wells assayed	Change in absorbance ($\times 10^3$) in 5 min at 610 nm		
		Standard assay	Standard assay with prior dicoumarol addition	Standard assay minus menadione
Control	16	212 \pm 12*	16.1 \pm 4.4	22.0 \pm 2.8
β -Naphthoflavone (2 μ M)	8	862 \pm 20	17.3 \pm 4.0	17.3 \pm 4.0
1,2-Dithiol-3-thione (10 μ M)	8	453 \pm 20	20.3 \pm 3.4	20.3 \pm 3.4

Note. Hepa 1c1c7 cells were grown and induced in three parallel sets of microtiter wells as described under Materials and Methods. One set of plates was lysed and assayed in the standard fashion, another set was assayed in the presence of 50 μ l of 0.3 mM dicoumarol per well, and the third set was assayed with reaction mixture containing no menadione.

* Mean values \pm standard deviations.

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ices were scanned 5 min after the reaction mixture. Since some quercers or crude fractions were used for inducer activity, it is desirable to have a quinone reductase assay of cells or the amount of inducer well. This normally is accomplished by microtiter plates treated with the MTT assay. We have used a slight modification of Drysdale *et al.* The media were deo-submerged in a vat of 2% ethanol for 10 min with tap water, and the plates were incubated at 30 °C for 24 h, refed with

-3-THIONE-TREATED

n 5 min at 610 nm

with rol	Standard assay minus menadione
	22.0 ± 2.8
	17.3 ± 4.0
	20.3 ± 3.4

well as described under other set was assayed in the reaction mixture containing no

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TABLE 2

COMPARISON OF QUINONE REDUCTASE INDUCTIONS OBTAINED BY DIRECT ASSAY OF HEPA 1c1c7 CELLS GROWN IN MICROTITER WELLS AND BY CONVENTIONAL ASSAY OF HEPA 1c1c7 CELLS GROWN ON 75-cm² PLATES

Compounds	Concentration (μM)	Ratio quinone reductase specific activity (treated/control)	
		Direct assay in microtiter wells ^a	Conventional assay with 75-cm ² plates ^b
Polycyclic aromatics			
2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin	0.01	3.10 ± 0.51 ^c	2.95 ± 0.40 ^d
β-Naphthoflavone	2	4.66 ± 0.25	3.56 ± 0.34 ^d
Benzo[<i>a</i>]pyrene	2	3.80 ± 0.34	3.58 ± 0.08 ^d
3-Methylcholanthrene	2	3.43 ± 0.40	3.29 ± 0.24 ^d
Azo dyes			
1,1'-Azonaphthalene	2	5.08 ± 0.36	4.47 ± 0.52 ^d
1-(2-Pyridylazo)-2-naphthol	2	4.80 ± 1.07	3.61 ± 0.26 ^d
1-(2-Thiazolylazo)-2-naphthol	2	3.23 ± 0.34	3.00 ± 0.18 ^d
Sudan I	2	4.25 ± 0.31	3.36 ± 0.12 ^d
Sudan II	2	2.90 ± 0.20	2.54 ± 0.20 ^d
Sudan III	2	1.80 ± 0.08	2.28 ± 0.20 ^d
Diphenols			
Catechol	30	1.98 ± 0.11	1.79 ± 0.20 ^d
Resorcinol	30	1.09 ± 0.08	0.88 ± 0.04 ^d
Hydroquinone	30	2.35 ± 0.19	1.92 ± 0.12 ^d
<i>tert</i> -Butylcatechol	30	1.75 ± 0.17	1.65 ± 0.10 ^d
<i>tert</i> -Butylresorcinol	30	0.97 ± 0.08	0.79 ± 0.08 ^d
<i>tert</i> -Butylhydroquinone	30	2.66 ± 0.23	2.87 ± 0.38 ^d
Isothiocyanate			
Benzylisothiocyanate	5	1.91 ± 0.20	3.16 ± 0.62 ^d
Dithiolthiones			
1,2-Dithiol-3-thione	10	2.65 ± 0.25	2.95 ± 0.40 ^d
4-Phenyl-1,2-dithiol-3-thione	30	3.53 ± 0.34	3.46 ± 0.56 ^d
5-(2-Pyrazinyl)-4-methyl-1,2-dithiol-3-thione	30	1.59 ± 0.14	2.32 ± 0.34 ^d

^a Direct assay described under Materials and Methods (*N* = 8).

^b Hepa 1c1c7 cells grown, treated, and assayed from 75-cm² plates as described by DeLong *et al.* (25).

^c Mean value ± standard deviation.

^d Unpublished results (*N* = 4).

^e From Prochaska *et al.* (24).

medium, and grown for an additional 24 h. The total cellular protein of each well of one plate was determined. The wells in this plate were washed with phosphate-buffered saline, 400 μl of water was added, and the wells were sonicated. Aliquots from each well were assayed by the method of Bradford (28), with bovine serum albumin as standard. A second plate was used to determine cell number per

well, and the remaining four plates were stained with crystal violet and destained as described for the 96-well plates. The stain from each well was solubilized in 3 ml of 0.5% sodium dodecyl sulfate in 50% ethanol and the absorbance of the resulting solution was measured in 1.0-cm light path cuvettes at 610 nm. The average absorbance for every concentration of cells from each individual

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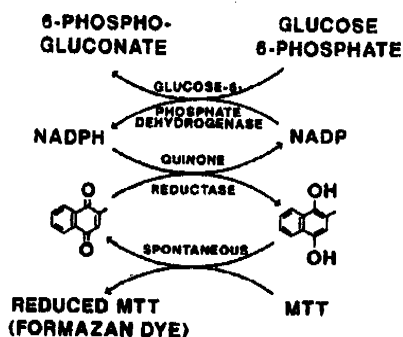


FIG. 1. Principle of the assay of quinone reductase. Glucose 6-phosphate and glucose-6-phosphate dehydrogenase continually generate NADPH, which is used by quinone reductase to transfer electrons to menadione. The menadiol reduces MTT to the blue formazan which can be measured over a broad range of wavelengths (550–640 nm). The complete reaction mixture is described under Materials and Methods. Both NADPH and menadione are regenerated, which obviates problems encountered with substrate depletion.

plate was used to determine the mean and standard error of absorbances shown in Fig. 4.

Determination of specific activities. The results of quinone reductase specific activity measurements for Table 2 are reported as the ratios of specific activities of inducer-treated microtiter wells to those of controls. The rate of MTT reduction and the crystal violet absorbances for the inducer-treated groups were compared to those of control cells grown on the same microtiter plates. The results were calculated using a spread-sheet program and the standard deviations shown in Table 2 were determined from the standard deviations of both the MTT and crystal violet assays.

RESULTS AND DISCUSSION

The assay (Fig. 1) is based on the production of a blue color when MTT is reduced nonenzymatically by menadiol that is generated enzymatically from menadione by quinone reductase. Similar systems have been

used for staining quinone reductase activity in gels (29). Although the bleaching of the color of 2,6-dichloroindophenol (a substrate for quinone reductase) by reduced nicotinamide nucleotides can be followed in microtiter wells, its use is unsatisfactory in this assay system for two main reasons. First, the depletion of 2,6-dichloroindophenol results in the significant decline of reaction rate with time. Second, small errors in pipetting of the reaction mixture containing 2,6-dichloroindophenol (which has an absorbance of 1.8 to 2.0 under usual assay conditions) can result in significant variability. These errors adversely affect the reproducibility of data since only the *absolute* absorbance at 5 min rather than the absorbance *change* in each well can be conveniently measured. The use of MTT reduction avoids these difficulties since (a) the menadione concentration remains constant in the assay system because MTT reduction results in menadione regeneration and (b) the assay depends on the generation of color from absorbances that are initially negligible. Thus, all wells have negligible absorbance at zero time and the absolute ab-

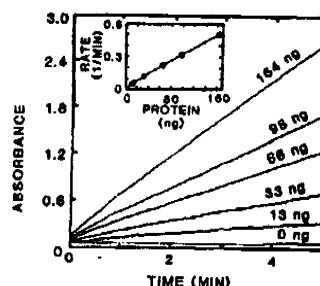


FIG. 2. Dependence of MTT reduction rate on amount of quinone reductase. Pure murine quinone reductase (30) was added in the indicated quantities and the rates of MTT reduction were recorded for 5 min at 610 nm in 1.0-cm light path cuvettes and in total volumes of 3.0 ml. The reaction mixture was identical to that used for microtiter well assays. Menadione was added to initiate the reaction. The assay was linear for 5 min for absorbance changes of up to 0.5/min. The rates obtained were proportional to the amount of enzyme added (inset; $r = 0.999$).

sorbance at 5 min change in absorbance extinction coefficient found to be 11,300.

The assay utilizes a system that maintains concentration, which is desired. Because the number of H₂O₂ grown in a micro measure convenient concentration is saturated ($\approx \frac{1}{2} K_m$; for this assay because promoted by other genases that are not able nor menadiol use of NADH res rates. With NAD⁺ dione-independent reduction of M less than 10% of the the standard assay of reduction of M⁺ diaphorases) is in presence of dicoumarol menadione, and it of the specific NAD⁺ Because the basal by NADPH is low parallel dicoumarol routine screening.

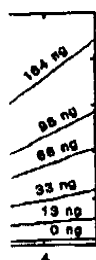
Since both substrate absorbance is line amount of added absorbance change of rate of absorbance enzyme is 0.001/min of MTT reduction microtiter wells is 1 of NADH oxidase standard menadiol chaska and Talalay of dicoumarol (50), the cuvette under used for the microtally instantaneous reduction. The rapid

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uctase activity eaching of the ol (a substrate duced nicotin- ured in micro- factory in this sons. First, the phenol results ction rate with ipetting of the 2,6-dichloroin- bance of 1.8 to ons) can result se errors ad- ry of data since at 5 min rather each well can e use of MTT lties since (a) remains con- ause MTT re- e regeneration the generation at are initially : negligible ab- e absolute ab-



duction rate on urine quinone- reated quantities and ured for 5 min at : and in total vol- e was identical to . Menadione was ay was linear for 5).5/min. The rates ount of enzyme

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sorbance at 5 min accurately reflects the change in absorbance during this time. The extinction coefficient of reduced MTT was found to be $11,300 \text{ M}^{-1} \text{ cm}^{-1}$ at 610 nm.

The assay utilizes an NADPH generating system that maintains a constant NADPH concentration, which can be varied as desired. Because the rate of MTT reduction by the number of Hepa 1c1c7 cells normally grown in a microtiter well is too rapid to measure conveniently if the NADPH concentration is saturating, $24 \mu\text{M}$ NADPH was chosen ($\approx K_m$; (30)). NADH is unsuitable for this assay because MTT reduction is also promoted by other NADH-linked dehydrogenases that are neither dicoumarol inhibitable nor menadione dependent. Thus, the use of NADH results in high nonspecific rates. With NADPH, however, the menadione-independent or dicoumarol-insensitive reduction of MTT with control cells was less than 10% of the total activity obtained in the standard assay (Table 1). This slow rate of reduction of MTT (caused by nonspecific diaphorases) is almost the same in both the presence of dicoumarol and in the absence of menadione, and it is unaffected by induction of the specific NAD(P)H:quinone reductase. Because the basal rate of reduction of MTT by NADPH is low, there is no need to run parallel dicoumarol-inhibited plates during routine screening.

Since both substrates are regenerated, the absorbance is linearly proportional to the amount of added enzyme up to a rate of absorbance change of 0.5 per min (Fig. 2). The rate of absorbance change in the absence of enzyme is 0.001/min. Furthermore, the rate of MTT reduction in the assay utilized for microtiter wells is linearly related to the rate of NADH oxidation as measured in the standard menadione assay described by Prochaska and Talalay (30; Fig. 3). The addition of dicoumarol ($50 \mu\text{M}$ final concentration) to the cuvette under conditions similar to those used for the microtiter assay results in virtually instantaneous inhibition of MTT reduction. The rapidity of inhibition is to be

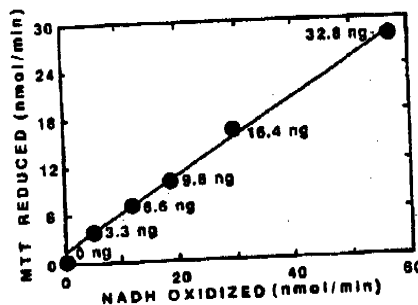


FIG. 3. Comparison of quinone reductase activity measured by the NADPH-dependent menadiol-mediated reduction of MTT with the standard quinone reductase assay. Six amounts of pure quinone reductase (30) indicated in the figure were assayed in the standard assay for quinone reductase as described by Prochaska and Talalay (30) by following the oxidation of NADH ($200 \mu\text{M}$) by menadione ($50 \mu\text{M}$) at 340 nm, as well as in the assay system utilized for microtiter wells by following the reduction of MTT by NADPH at 610 nm. Both assays were performed at 25°C in 1.0-cm light path cuvettes and in total volumes of 3.0 ml. The rates obtained by the two assays are linearly correlated ($r = 0.998$) and the MTT assay rate was 46.7% that of the standard NADH assay.

expected since the K_i value for dicoumarol is low (110 nM) and the concentration of the competing substrate (NADPH) is also low ($20 \mu\text{M}$) (30). Thus, this system provides an appropriate assay for quinone reductase.

Because we were interested in maximizing the rapidity with which the screening of inducers could be performed, we modified the method of crystal violet staining described by Drysdale *et al.* (27). This procedure has been used with great success to determine the specific activity of cytotoxic factors in the L929 murine fibroblast line, since it is a rapid, simple, and reliable method for determining cell number (27). Staining with crystal violet also appears to be well suited for the Hepa 1c1c7 murine hepatoma cell line since the degree of crystal violet absorption correlates well with cell number and total protein (Fig. 4; $r = 0.996$ and 0.997 , respectively). Indeed, at exceedingly high cell densities/

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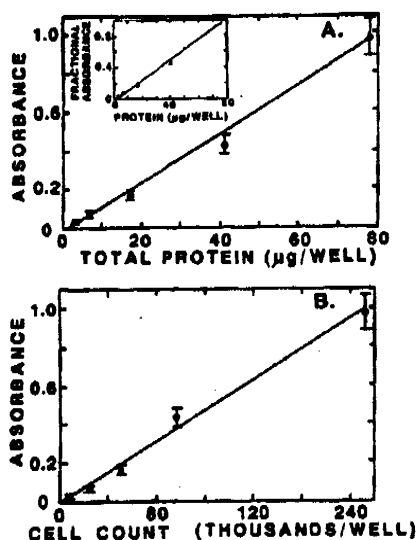


FIG. 4. Crystal violet staining correlates with total cellular protein (A) and cell number (B). Hepa 1c1c7 cells were plated at five cell densities in six identical 24-well 2-cm² plates and grown as described under Materials and Methods. Aliquots from one plate were used to estimate cell protein (presented as µg of protein per well) by the method of Bradford (28), and a second plate was used to determine cell number. The remaining four plates were stained, destained, and solubilized in 3 ml of 0.5% sodium dodecyl sulfate in 50% ethanol. The solutions were transferred to 1.0-cm light path cuvettes and the absorbances were then determined at 610 nm. The average crystal violet absorbance for every concentration of cells from each plate was determined, from which the mean absorbances and standard errors shown in the figure were calculated ($N = 4$). The absorbances of crystal violet were linearly correlated with total protein and cell number ($r = 0.997$ and 0.996 , respectively). Furthermore, the proportion of crystal violet absorbance relative to the highest absorbance was virtually the same between plates (inset).

cm², the crystal violet absorbance continues to correlate well with total protein, although the ratio of crystal violet absorbances to cell number increases (data not shown). Although there is some variability of the absolute absorbance of crystal violet between individual plates, the relative proportion of

staining of cells of different cell densities is remarkably constant (Fig. 4, inset). Hence, we find that crystal violet staining is a suitable method for the rapid estimation of total cellular protein and/or cell number and the data from Fig. 4 can be used to derive a simple formula for estimating quinone reductase specific activity.³ We found that over many ($N = 32$) experiments the specific activities ranged between 104 ± 3.4 and 355 ± 18.7 nmol/min/mg, and the mean \pm standard deviation of the averages is 208 ± 66 nmol/min/mg. The specific activity of quinone reductase in Hepa 1c1c7 cells grown in 75-cm² plates and assayed in the conventional manner with menadione ($50 \mu\text{M}$) and NADH ($200 \mu\text{M}$) as substrates ranged from 213 ± 6.6 to 578 ± 81.6 nmol/min/mg of protein. The mean and standard deviation of the averages

³ The specific activity of quinone reductase (nmol/min/mg of protein) can be estimated by using the extinction coefficient of MTT ($11,300 \text{ M}^{-1} \text{ cm}^{-1}$ at 610 nm) and the measure of total cellular protein as determined by the proportionality constant calculated from the calibration curve for crystal violet. This constant (37.8 ml/mg/cm light path at 610 nm) is the slope of the line depicted in Fig. 4A multiplied by 3.0 ml (the volume in which the crystal violet stain was solubilized). Because of the orientation of the light beam relative to the microtiter well, the absorbance of a given quantity of chromophore is independent of volume; i.e., the product of the concentration and path length is a constant. In other words, for a given amount of chromophore, if the concentration is halved by the addition of solvent, the path length is doubled and the total absorbance remains unchanged. Thus, the moles of MTT reduced or the mass of protein per well can be determined from their respective absorbances, the extinction coefficient or proportionality constant, and the area of the microtiter well (0.32 cm^2). Furthermore, since both the MTT and crystal violet assays are scanned in microtiter wells of the same diameter, the specific activity calculation becomes independent of area. Therefore, specific activity can be calculated from the simple formula

specific activity

$$= \frac{\text{absorbance change of MTT/min}}{\text{absorbance of crystal violet}} \times 3345 \text{ nmol/mg}$$

where 3345 nmol/mg is the ratio of the proportionality constant determined for crystal violet and the extinction coefficient of MTT.

BLANK —
CONTROL —
SUDAN I —
SUDAN II —
SUDAN III —

FIG. 5. Photographs of the assay wells in the assays. Hepa 1c1c7 cells grown in 75-cm² plates and assayed in the conventional manner with menadione ($50 \mu\text{M}$) and NADH ($200 \mu\text{M}$) as substrates ranged from 213 ± 6.6 to 578 ± 81.6 nmol/min/mg of protein. The mean and standard deviation of the averages

of 15 experiments of protein.

The usefulness of screening for induction is illustrated in Table 1, which shows that accurately identified inducers and yield order of induction of cells grown on 75-cm² plates. The conventional assay system have reported that the induction of quinone reductase by hydroquinones levels of quinone reductase in Hepa 1c1c7 cell line (observed with the direct assay system) rank order of induction tested in the direct assay system. The conventional assay system demonstrates that the induction can be

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it cell densities is 4, inset). Hence, staining is a suitable estimation of total number and the direct assay of quinone reductase activity that over many specific activities and 355 ± 18.7 nmol/min \pm standard deviation of quinone reductase activity of quinone reductase grown in 75-cm² conventional manner (μ M) and NADH from 213 ± 6.6 ng of protein. The mean of the averages

one reductase (nmol/min) calculated by using the extinction coefficient of 610 nmol protein as determinant calculated from violet. This constant (nm) is the slope of the linear relationship between the absorbance and the volume of the solution. Because the absorbance is relative to the amount of chromophore, i.e., the product of the path length and the concentration of the chromophore, if the concentration of the chromophore remains unchanged or the mass of the chromophore is reduced from their respective extinction coefficient or proportion of the microtiter well in the MTT and crystal violet wells of the calculation becomes specific activity can be

$\frac{1}{\text{mg}} \times 3345 \text{ nmol/mg}$

the proportionality of the extinction

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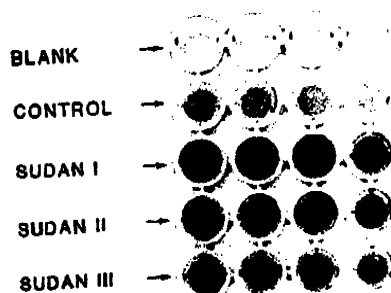


FIG. 5. Photograph showing the color (blue) that develops in the assays for quinone reductase activity of Hepa 1c1c7 cells grown in microtiter wells. The cells were grown, induced, and assayed as described under Materials and Methods. Blank wells contain no cells; control wells contain Hepa 1c1c7 cells treated with medium containing 0.1% DMSO (without inducer). Sudan I, II, and III wells contain cells that were treated with media containing the respective azo dye (2 μ M) in 0.1% DMSO. All cells were grown for 24 h and then treated with inducer or DMSO for another 24 h prior to assay. Four identical wells are shown for each condition.

tance of a microtiter scanner. Scanning of the absorbances for the experiment shown in Table 2 required less time than did harvesting of cells from the equivalent number of 75-cm² plates. Data processing can be further simplified by linking the scanner to a personal computer. We conclude that the direct assay of quinone reductase from cells grown in microtiter wells may facilitate the identification and isolation of novel inducers of chemoprotective enzymes such as quinone reductase.

ACKNOWLEDGMENTS

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of 15 experiments is 357 ± 106 nmol/min/mg of protein.

The usefulness of the microtiter system in screening for inducers of quinone reductase, is illustrated in Table 2 and Fig. 5. This assay accurately identified inducers and noninducers and yielded virtually the same rank order of induction as did experiments with cells grown on 75-cm² plates and assayed in the conventional manner. For example, we have reported that resorcinol and its substituted congeners were inactive as inducers of quinone reductase, whereas catechols and hydroquinones could significantly elevate levels of quinone reductase in the Hepa 1c1c7 cell line (24). The same patterns were observed with the diphenols tested in the direct assay system (Table 2). Furthermore, the rank order of induction potency of azo dyes tested in the direct assay is the same as in the conventional assay system. Figure 5 demonstrates that the degree of quinone reductase induction can be detected without the assis-

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